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THE STRUCTURE OF THE ALDOBIONIC ACID FROM FLAXSEED MUCILAGE

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Indications of the presence of an aldobionic acid in the hydrolysate from flaxseed (linseed) mucilage were first obtained by Hilger¹ in 1903, and later confirmed by Neville.²

Anderson and Crowder³ succeeded in isolating the aldobionic acid, as its calcium and barium salts, and found it to be a compound of galacturonic acid with *l*-rhamnose. The *l*-rhamnose was definitely identified but the other moiety was only recognized as galacturonic acid. It remained for Niemann and Link⁴ to prove conclusively that it was actually the *d* form of the uronic acid.

By oxidation with hypiodite, Anderson and Crowder transformed the aldobionic acid to the corresponding dibasic acid, which still contained uronic acid (as revealed by the naphthoresorcinol test), and they were therefore able to assign to the aldobionic acid the structure of a galacturonido-*l*-rhamnose, tentatively suggesting that the biose link is at position 4 of the rhamnose residue, since position 6 is excluded and other positions of combination were unknown at that time.

Examples of linkage at positions 2, 3, and 5 are now known⁵⁻⁷

¹ Hilger, A., *Ber. chem. Ges.*, **36**, 3197 (1903).

² Neville, A., *J. Agric. Sc.*, **5**, 113 (1913); *Chem. Trade J.*, 265 (1911).

³ Anderson, E., and Crowder, J. A., *J. Am. Chem. Soc.*, **52**, 3711 (1930).

⁴ Niemann, C., and Link, K. P., *J. Biol. Chem.*, **104**, 205 (1934).

⁵ Hirst, E. L., and Jones, J. K. N., *J. Chem. Soc.*, 1174 (1938).

⁶ Percival, E. G. V., and Somerville, J. C., *J. Chem. Soc.*, 1615 (1937).

⁷ Haworth, W. N., Raistrick, H., and Stacey, M., *Biochem. J.*, **31**, 640 (1937).

to occur in natural substances built up of sugar or uronic acid residues, or of both types of residue. Further investigation of the structure of the aldobionic acid was therefore desirable.

The aldobionic acid was prepared from flaxseed mucilage by an improvement of the method of Anderson and Crowder,³ and the product then completely methylated to the methyl ester of the pentamethyl methylaldobionide. This fully methylated derivative consisted of a syrupy mixture of α and β forms of the glycoside (*i.e.*, of the rhamnose moiety), but it was possible to isolate one form as crystalline material having a melting point of 93–94°.

On hydrolysis with mineral acid, the syrupy and the crystalline portions gave rise to equimolecular proportions of the same two products; namely, a crystalline trimethyl galacturonic acid and a crystalline dimethyl rhamnose.

The methylated uronic acid was recognized as 2,3,4-trimethyl *d*-galacturonic acid⁸ since, on condensation with methanol containing dry hydrogen chloride, it yielded the crystalline methyl ester of 2,3,4-trimethyl α -methyl-*d*-galacturonide previously described.⁹

It seemed plausible that the dimethyl rhamnose moiety might be either 2,3- or 3,4-dimethyl rhamnose. Negative evidence, suggesting the latter as the structure, was derived from the observation that the sugar showed no change in specific rotation on preservation of a solution of it in methanol containing 1 per cent of dry hydrogen chloride, during 20 hours at room temperature.

The identity of the dimethyl rhamnose was established by comparison of its properties with those of an authentic specimen of the 3,4-dimethyl *l*-rhamnose previously known.¹⁰ They had the same specific rotation and melting point, and a mixture of the two showed no depression of melting point. The identification was confirmed by transformation of the dimethyl rhamnose (from flaxseed mucilage) to the corresponding lactone. This was the same as the 3,4-dimethyl δ -rhamnonolactone previously known.¹⁰

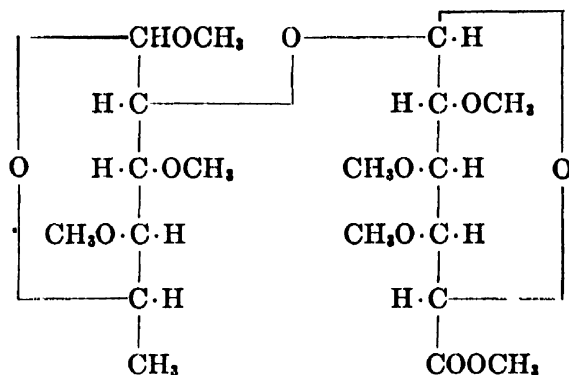
⁸ Tipson, R. S., *J. Biol. Chem.*, **125**, 341 (1938).

⁹ Levene, P. A., and Kreider, L. C., *J. Biol. Chem.*, **120**, 597 (1937).
Levene, P. A., Tipson, R. S., and Kreider, L. C., *J. Biol. Chem.*, **122**, 199 (1937–38).

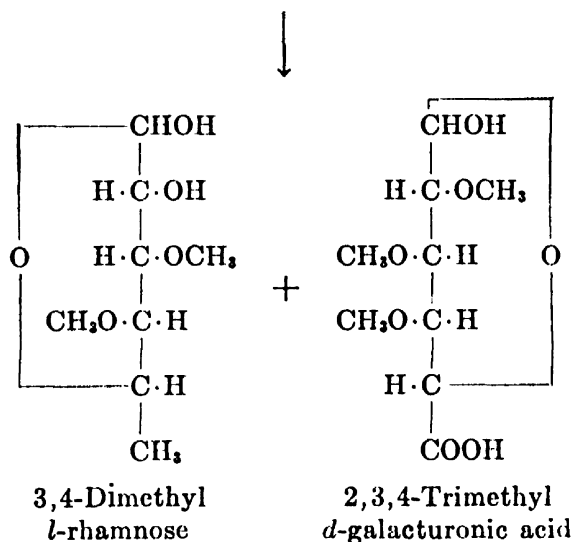
¹⁰ Haworth, W. N., Hirst, E. L., and Miller, E. J., *J. Chem. Soc.*, 2469 (1929).

Further oxidation, with nitric acid, led to a dimethoxy hydroxyglutaric acid (isolated as its crystalline dimethylamide), analogous to that described by Baker and Haworth¹¹ as formed by nitric acid oxidation of trimethyl *l*-arabofuranose.

These observations clearly show that the uronic acid residue is the pyranose form of *d*-galacturonic acid and that, in the aldo-



Methyl ester of pentamethyl 2-*d*-galacturonido-methyl-*l*-rhamnoside



bionic acid, this is attached by a glycosidic link to position 2 of the rhamnose residue. The aldobionic acid from flaxseed mucilage may therefore be formulated as 2-(*d*-galacturonopyranosido)-*l*-rhamnose. Depending on the conditions, the rhamnose moiety is free to function either as a furanose or a pyranose.

¹¹ Baker, S., and Haworth, W. N., *J. Chem. Soc.*, **127**, 365 (1925).

EXPERIMENTAL

Preparation of Ash-Free Flaxseed Mucilage—The whole flaxseed was soaked in distilled water for 2 days and the mucilage then separated from the seed by pressing through a wire sieve (No. 16 mesh). The mucilage was now filtered through fine cheese-cloth in order to remove particles of whole seed, precipitated with alcohol, squeezed free of solution, and soaked in alcohol until no longer sticky. The product contained a large amount of ash which was removed by the following treatment.

The dried, stringy, tough substance was dissolved in boiling 1 per cent hydrochloric acid, the solution allowed to stand during 15 minutes at room temperature, and the material then precipitated by the addition of 3 volumes of absolute ethyl alcohol. The product was a fine amorphous powder (no longer stringy), which was filtered off and dried with absolute alcohol and dry ether. The average yield from 50 pounds of whole flaxseed was about 750 gm.

Hydrolysis of Ash-Free Flaxseed Mucilage with Dilute Sulfuric Acid—A mixture of 1.5 kilos of dry, powdered, ash-free flaxseed mucilage with 1 liter of charcoal was added to 12 liters of 4 per cent sulfuric acid and the suspension was heated on a steam bath during 9 hours. The hot mixture was then treated with hot barium hydroxide solution until no longer acid to Congo red but acid to blue litmus. The remainder of the acid was neutralized with barium carbonate. The mixture was now filtered and the precipitate stirred for 1 hour with 8 liters of hot water and the suspension filtered.

The combined filtrates were concentrated to 1.5 liters and then precipitated, with vigorous stirring, by slowly adding 3 liters of absolute ethyl alcohol (all mother liquors being saved). The precipitated syrup was dissolved in 1 liter of water and partially precipitated with 500 cc. of alcohol. The black, tarry, precipitated syrup was discarded and the material in the mother liquor precipitated with 2 liters of alcohol. The syrup was reprecipitated by dissolving in 1 liter of water and adding 2 liters of alcohol.

This purified syrup was dissolved in 500 cc. of water, stirred overnight with charcoal, filtered, and the filtrate poured into 2.5 liters of alcohol. The precipitated amorphous powder was cen-

trifuged off, shaken well with absolute alcohol, and again centrifuged. The product was stirred for 1 hour with 1 liter of absolute alcohol, filtered, washed successively with absolute alcohol and dry ether, and then dried for 24 hours in the vacuum oven at 80°. Yield 105 gm. A Willstätter estimation gave an average molecular weight of 470 to 480. ($C_{12}H_{19}O_{11}Ba_4$, calculated 408.)

The aqueous alcohol mother liquors from the above material were concentrated to dryness, dissolved in 500 cc. of water, and precipitated with 1.5 liters of alcohol. This syrup was precipitated twice more in the same manner and then dissolved in 300 cc. of water and poured into 1500 cc. of alcohol. The product was now isolated as above, giving a second crop of 55 gm. of the barium salt having an average molecular weight of 450 to 460 on the basis of a Willstätter estimation.

These two fractions were then combined and used in the subsequent experiments.

When the ash-free flaxseed mucilage was hydrolyzed during 18 to 20 hours with 4 per cent sulfuric acid according to the directions of Anderson and Crowder,³ a barium salt was obtained which had a molecular weight (by Willstätter's method) of 410, which is close to the calculated value (408). However, when this material was methylated as now described, it was found to contain 60 to 70 per cent of monose, whereas the material obtained after hydrolysis during 9 hours contained only 10 to 15 per cent of monose. The Willstätter method evidently does not give the correct value in the case of this substance and treatment during 18 to 20 hours hydrolyzes the aldobionic acid to a great extent.

Methyl Ester of Pentamethyl Methylaldobionide from Flaxseed Mucilage—15 gm. of the barium salt of the aldobionic acid from flaxseed mucilage were dissolved in 50 cc. of water and almost quantitatively freed from barium with sulfuric acid. Care was taken to leave about 2 to 3 per cent of the barium in order to avoid the presence of free sulfuric acid. The barium sulfate was removed and the solution concentrated to dryness. The free aldobionic acid was separated from the small amount of barium salt by extracting with warm methanol, filtering, and evaporating the filtrate to dryness.

The free acid was methylated with sodium hydroxide and dimethyl sulfate according to the directions of Levene and co-

workers.¹² Yield 6.1 gm. The substance was then esterified with diazomethane, methylated five times with Purdie's reagents, and the product distilled at a pressure of 0.1 to 0.2 mm. A small portion (0.5 gm.) of low boiling methylated monose derivative was obtained at 130-135°, while the main product (4.1 gm.) distilled at 165-169° (bath temperature, 170-175°). This main product was then redistilled and had a composition agreeing approximately with that calculated for the methyl ester of a pentamethyl hexuronido-methylmonodesoxyhexoside.

2.805 mg. substance: 27.08 cc. 0.01 N sodium thiosulfate
 $C_{19}H_{34}O_{11}$. Calculated, OCH_3 49.55; found, OCH_3 49.91

This glassy syrup was dissolved in 30 to 40 cc. of hexane and, upon slow spontaneous evaporation, the substance crystallized. Total yield of crystals, 1.4 gm. The crystalline product had a composition agreeing with that calculated for the methyl ester of a pentamethyl hexuronido-methylmonodesoxyhexoside.

5.670 mg. substance: 10.790 mg. CO_2 and 3.990 mg. H_2O
 3.227 " " : 30.92 cc. 0.01 N sodium thiosulfate
 $C_{19}H_{34}O_{11}$. Calculated. C 52.02, H 7.8, OCH_3 49.55
 Found. " 51.90, " 7.8, " 49.54

The substance was recrystallized many times from hexane but a constant melting point was never obtained, although the composition remained unchanged.

The crystalline methyl ester of pentamethyl methylaldobionide had a rather indefinite melting point at about 93-94° and the following specific rotation.

$$[\alpha]_D^{25} = \frac{+2.93^\circ \times 100}{2 \times 1.129} = +129.8^\circ \quad (\text{in water})$$

The syrupy methyl ester had $n_D^{25} = 1.4675$.

Hydrolysis of Methyl Ester of Pentamethyl Methylaldobionide—The course of hydrolysis at 100° of a 2 per cent solution of the crystalline methyl ester of pentamethyl methylaldobionide in 7 per cent aqueous hydrochloric acid was studied polarimetrically. Under these conditions, the initial specific rotation of the solution

¹² Levene, P. A., Meyer, G. M., and Kuna, M., *J. Biol. Chem.*, **125**, 703 (1938). Levene, P. A., and Kuna, M., *J. Biol. Chem.*, **127**, 49 (1939).

($[\alpha]_D^{25} = +130.0^\circ$) showed a smooth change as follows: $+119.6^\circ$ (30 minutes), $+111.2^\circ$ (1 hour), $+97.0^\circ$ (2 hours), $+86.4^\circ$ (3 hours), $+78.5^\circ$ (4 hours), $+73.4^\circ$ (5 hours), $+71.3^\circ$ (6 hours), constant thereafter. (The rotation of an equimolecular mixture of 2,3,4-trimethyl galacturonic acid⁸ and 3,4-dimethyl rhamnose¹⁰ is calculated to be $+70^\circ$.)

The solution, from 4 gm. of methylglycoside methyl ester, was cooled to room temperature and the hydrochloric acid neutralized by addition of silver carbonate. The precipitate was centrifuged off and washed several times by shaking with water and centrifuging.

The solution and washings were combined and hydrogen sulfide was passed into the solution until all the silver had been precipitated. The mixture was then aerated, filtered, and the filtrate evaporated under diminished pressure to a volume of 125 cc.

A slight excess of barium carbonate was now added and the mixture was warmed at 60° until the solution was neutral to litmus. The mixture was then filtered and the filtrate evaporated under diminished pressure to a yellow glass (weight, 4.5 gm.). This was thoroughly extracted with fifteen portions (100 cc. each) of boiling dry ether under a reflux condenser. The ether extracts were united and evaporated to dryness under diminished pressure, giving a colorless crystalline mass (weight, 1.5 gm.). This crystalline material was recrystallized from ether-pentane and then had a melting point of $94-95^\circ$, $n_D^{25} = 1.4711$ (in the superfused state), and the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.36^\circ \times 100}{2 \times 0.990} = +18.2^\circ \quad (\text{equilibrium, in water})$$

It had the following composition.

5.402 mg. substance:	9.890 mg. CO ₂ and 4.070 mg. H ₂ O
3.889 " " "	: 24.22 cc. 0.01 N sodium thiosulfate
C ₈ H ₁₆ O ₆ .	Calculated. C 49.97, H 8.4, OCH ₃ 32.30
	Found. " 49.92, " 8.4, " 32.20

The properties previously recorded¹⁰ for authentic 3,4-dimethyl rhamnose are, m.p. = $91-92^\circ$ and $[\alpha]_D^{20} = +18.6^\circ$ (equilibrium, in water).

In methyl alcohol containing 1 per cent of dry hydrogen chloride

the sugar had $[\alpha]_D^{22} = -8.8^\circ$ ($c = 1.02$), and this value remained constant during several hours at room temperature, indicating that there was no free hydroxyl group at carbon atom (4).

After removal of the dimethyl rhamnose, by extraction with ether, the residue consisted of the barium salt of trimethyl galacturonic acid. This material was dissolved in water and the barium removed quantitatively by adding N sulfuric acid dropwise.

The barium sulfate was removed by centrifuging, the precipitate washed several times by centrifuging with water, and the combined solutions evaporated under diminished pressure to a volume of 25 cc. This solution was then extracted with four portions (50 cc. each) of chloroform, the chloroform extract dried with anhydrous sodium sulfate, filtered, and the filtrate evaporated to a colorless glass, which was obtained crystalline from ether.

As the product was difficult to purify, it was identified by transformation to its methylglycoside methyl ester.

Preparation of Methyl Ester Methylglycoside of Trimethyl Galacturonic Acid—A 1 per cent solution of the trimethyl galacturonic acid in methyl alcohol containing 1 per cent of dry hydrogen chloride was gently boiled under a reflux during 6 hours.

The solution was then cooled and rendered neutral by the addition of dry silver carbonate. The mixture was filtered and the filtrate evaporated to dryness under diminished pressure, giving a quantitative yield of colorless, glassy substance. It was readily obtained crystalline by dissolving in dry ether, filtering off a trace of insoluble material, and evaporating the filtrate in a dish at room temperature. It separated in large square platelets which were filtered off, washed with a little ether, and dried. M.p. = 70° .

It had the following composition.

5.201 mg. substance: 9.510 mg. CO_2 and 3.570 mg. H_2O

2.800 " " : 31.73 cc. 0.01 N sodium thiosulfate

$C_{11}H_{20}O_7$. Calculated. C 49.97, H 7.6, OCH_3 58.72
Found. " 49.87, " 7.7, " 58.59

Its specific rotation was as follows:

$$[\alpha]_D^{24} = \frac{+3.58^\circ \times 100}{2 \times 1.202} = +149.0^\circ \quad (\text{in acetone})$$

The properties previously recorded⁹ for the methyl ester of 2,3,4-trimethyl α -methyl-*D*-galacturonide are m.p. = 70.2–70.3° and $[\alpha]_D^{27} = +149.3^\circ$ (in acetone).

Oxidation of Dimethyl Rhamnose (from Aldobionic Acid) with Bromine—To a solution of 0.5 gm. of crystalline dimethyl rhamnose in 10 cc. of water was added 0.5 cc. of bromine. The mixture was kept in a glass-stoppered Erlenmeyer flask during 24 hours at room temperature. The brown solution was then aerated until colorless and sufficient silver carbonate was added to neutralize all the mineral acid. The mixture was filtered, the precipitate washed with water, and excess hydrogen sulfide passed into the combined filtrate and washings. The mixture was aerated, filtered, and the filtrate evaporated to dryness. The product was dissolved in dry ether, filtered from a trace of insoluble material, and the filtrate evaporated to dryness, giving a colorless, crystalline mass.

It was recrystallized by dissolving in 5 cc. of dry ether, adding 20 cc. of pentane, and allowing to stand in the refrigerator, whereupon it separated as a mat of long, fine colorless needles having a melting point of 76–77° and the following specific rotation.

$$[\alpha]_D^{24} = \frac{-0.71^\circ \times 100}{2 \times 0.224} = -158.5^\circ \quad (\text{initial, in water})$$

changing to -120.5° (50 hours), and -116.1° (92 hours), constant thereafter.

It had the following composition.

5.514 mg. substance:	10.195 mg. CO ₂ and 3.600 mg. H ₂ O
3.299 " " "	: 20.80 cc. 0.01 N sodium thiosulfate
C ₈ H ₁₄ O ₅ .	Calculated. C 50.50, H 7.4, OCH ₃ 32.64
	Found. " 50.43, " 7.3, " 32.59

The properties previously recorded¹⁰ for 3,4-dimethyl δ -rhamnonolactone are m.p. = 66–68° and $[\alpha]_D^{26} = -153^\circ$ (initial value, in water), changing to the constant value of -119° after 86.5 hours.

Oxidation of Dimethyl Rhamnose (from Aldobionic Acid) with Nitric Acid—0.45 gm. of crystalline dimethyl rhamnose (from flaxseed mucilage) was dissolved in 8 cc. of concentrated nitric acid ($d = 1.42$) and the oxidation conducted exactly as previously

described for the oxidation of trimethyl xylulose.¹³ The product was esterified and the ester treated with a solution of methylamine in methanol. Colorless crystals of the dimethylamide were obtained, having the following composition.

2.762 mg. substance: 4.705 mg. CO₂ and 1.900 mg. H₂O
3.300 " " : 0.332 cc. N₂ (768 mm. at 27°)
C₆H₁₄O₆N₂. Calculated. C 46.12, H 7.8, N 11.97
Found. " 46.45, " 7.7, " 11.57

Preparation of 3,4-Dimethyl l-Rhamnose from l-Rhamnose—Since the yield at each stage of the preparation was greatly improved by appropriate modification of the methods of earlier workers, our method will be briefly described.

50 gm. of rhamnose hydrate were suspended in 200 cc. of dry pyridine. 200 cc. of acetic anhydride were added and after about 5 minutes at room temperature, the suspension was cooled somewhat in ice. After 15 minutes the sugar had dissolved completely. The solution was then cooled to 25° and allowed to stand overnight at room temperature.

The solution was now evaporated under diminished pressure to a syrup weighing 120 gm. This was poured, with stirring, into 1 liter of filtered ice water. The precipitated syrup was dissolved in chloroform and the aqueous layer extracted several times with chloroform. The combined chloroform solution was washed successively with ice-cold dilute sulfuric acid, ice water, dilute sodium bicarbonate solution, and ice water. It was dried with anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness, giving a practically quantitative yield of syrupy tetraacetyl rhamnose. (Previously recorded yields, 75 per cent,¹⁴ 80 to 85 per cent.¹⁰)

The tetraacetyl *l*-rhamnose was then converted to the bromotriacetyl derivative as described for the preparation of bromotetraacetyl mannose,¹⁵ giving a yield of 96.5 per cent of the theoretical of dry, crystalline product. (Previously recorded yields, 65 per cent,¹⁴ 85 per cent.¹⁰)

¹³ Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **120**, 607 (1937).

¹⁴ Fischer, E., Bergmann, M., and Rabe, A., *Ber. chem. Ges.*, **53**, 2362 (1920).

¹⁵ Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **90**, 89 (1931).

By the following method, the bromoacetate was transformed to 3,4-diacetyl 1,2-methyl-orthoacetyl rhamnose, a yield of 76.5 per cent of the theoretical of dry, crystalline product being readily obtained. (Previously recorded yields, 20 to 40 per cent,¹⁴ variable up to 50 per cent but usually about 20 per cent,¹⁰ 45 per cent.¹⁶)

To 10 gm. of dry, crystalline bromotriacetyl *l*-rhamnose was rapidly added a solution of 4 cc. of quinoline (dried over barium oxide) in 20 cc. of dry methanol, the mixture was rapidly swirled until all the crystals of bromoacetate had dissolved, and then the solution was kept at room temperature, with the exclusion of atmospheric moisture, during 90 minutes.

The solution was now diluted to 75 cc. with dry methanol and a considerable excess of silver acetate was added. The mixture was filtered with suction through a thin layer of silver acetate and the precipitate well washed with methanol. The combined filtrate and washings were evaporated to dryness under diminished pressure to a thick syrup, which was dissolved in chloroform, diluted to 1 liter with chloroform, and the precipitated silver acetate removed by filtration. Evaporation of the chloroform solution under diminished pressure gave a pale yellow syrup, which was dissolved in 10 cc. of warm methanol. 10 cc. of water were added to the solution and, on standing in the refrigerator, a first crop of 4 gm. of crystalline product was deposited.

The mother liquor was evaporated to dryness, dissolved in chloroform, and the chloroform solution shaken successively with water, dilute sodium bicarbonate solution, and water. It was dried with anhydrous sodium sulfate, filtered, and the filtrate evaporated to dryness. Ten separate portions (50 cc. each) of water were now added, the mixture being evaporated to dryness before each new addition. In this way, the major part of the free quinoline was removed.

The resulting syrupy product was now dissolved in a little dry ether and a large volume of pentane was added. On standing in the refrigerator, a second crop of 2.6 gm. of crystalline product settled out. The total yield of crystalline product amounted to 76.5 per cent of the theoretical.

¹⁶ Haworth, W. N., Hirst, E. L., and Samuels, H., *J. Chem. Soc.*, 2861 (1931).

The 3,4-diacetyl 1,2-methyl-orthoacetyl rhamnose was then deacetylated in the usual manner, with a solution of ammonia in methanol, to give a quantitative yield of the orthoacetate. The product was methylated and hydrolyzed as described by Haworth and coworkers¹⁰ to give 3,4-dimethyl rhamnose having a melting point of 94–95° and other properties as described by these authors.

DELPHININE

II. ON OXODELPHININE

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Analytical results obtained with delphinine, the crystalline alkaloid from *Delphinium staphisagria*, L., have been shown by us (1) to correspond best with a formula $C_{33}H_{45}O_9N$ for this alkaloid. Its assumed close relationship to the aconite group of alkaloids has now been clearly demonstrated. In addition to four methoxyl groups it contains a benzoyl, an acetyl, and a free acylatable hydroxyl group and behaves as if it contains an N-alkyl (methyl) group. More recent studies have confirmed this analogy to the aconite alkaloids, since it loses acetic acid on pyrolysis to form a *pyrodelphinine*. It is probable that the hydroxyl and acetyl groups occur in a 1-4 or 1-5 relationship and that acetic acid is lost with the formation of an oxidic bridge. This has been indicated by the hydrogenation experiments in which only the benzoyl group appears to be involved.

The study of the oxidation of delphinine with permanganate has been carried further since the earlier report. In addition to the formation of the substance first described by Keller (2) and called by him X-214°, we have isolated a second apparently isomeric substance formed in smaller amount. By a modification of the earlier procedure a total yield of roughly 90 per cent has been obtained of the two isomers. For the reasons given below, the preponderating substance has been called α -oxodelphinine ($[\alpha]_D^{20} = -62^\circ$ in acetic acid) and its companion β -oxodelphinine ($[\alpha]_D^{20} = +31^\circ$). Many analyses of these substances, including our earlier published figures, are consistent with a formulation $C_{33}H_{43}O_{10}N$. This is a conclusion which has an important bearing on the interpretation of the correct formulation of oxonitine and its isomer (?),

as discussed in the succeeding paper (3). Just as in the case of oxonitine and its companion, the isomeric oxodelphinines have lost the essential basic character of the parent alkaloid. Both the benzoyl and acetyl groups are retained, and on catalytic hydrogenation of the α isomer a *hexahydro- α -oxodelphinine* was formed. Although four methoxyl groups can still be readily determined, only a trace of an N-methyl could be detected. The above formulation $C_{33}H_{43}O_{10}N$, if correct, points to the oxidation of a CH_2 group adjoining the N atom to CO with the formation of a cyclic lactam. Either the so called N- CH_3 group is so modified by such a transformation that it eludes determination as an N- CH_3 group (4) or the so called N- CH_3 group of delphinine does not exist as such but as a larger grouping. The formation of methylamine from delphinine or methyl iodide during the N-alkyl determination would be difficult to explain except as the result of the cleavage of some larger grouping, such as a complex pyrrolidone, piperidone, or related derivatives. This is an inconsistency which can be cleared up only by further investigation.

The above formulation of α -oxodelphinine is supported by the analytical figures obtained with *pyro- α -oxodelphinine* formed from it on pyrolysis and with those given by *hexahydropyro- α -oxodelphinine* resulting from the latter on hydrogenation. When α -oxodelphinine was heated with methyl alcoholic HCl, a crystalline neutral substance resulted. The analytical figures are in approximation to those required by a formula $C_{32}H_{43}O_9N$. The methoxyl determination indicates introduction of an extra methoxyl group (a total of five OCH_3 groups) and titration after saponification the presence of only one acyl group. It appears, therefore, that the acetyl group has been replaced by methyl. Simultaneously an appreciable amount of a basic fraction was isolated from the mixture which, however, has not been obtained in crystalline form. Since CO_2 was evolved in the reaction, the formation of this base possibly parallels that of the base obtained in a similar manner from oxonitine, as described in the succeeding paper (3).

EXPERIMENTAL

Pyrodelphinine—0.57 gm. of delphinine was heated for 10 minutes in an atmosphere of H_2 at 200–215°. The melted alkaloid evolved acetic acid. The resulting resin crystallized readily under

ether and was collected in excellent yield. Recrystallized from 95 per cent alcohol, it formed pointed rods which slowly melted at 208–212° after preliminary softening. 0.28 gm. was so obtained. For analysis it was dried at 110° and 20 mm.

$C_{31}H_{41}O_7N$. Calculated, C 68.97, H 7.66; found, C 69.11, H 7.57

Pyrodelphinine readily absorbed H_2 in acetic acid solution with platinum oxide catalyst. After deducting for catalyst, the apparent absorption approximated 4 moles of H_2 . The resulting substance could not be obtained in crystalline form.

α - and β -Oxodelphinine—Since our preliminary report on this substance, the following improved method for its preparation has been employed.

5 gm. of delphinine were dissolved in 500 cc. of dry acetone containing 5 cc. of acetic acid. 3 gm. of powdered potassium permanganate were then added and the mixture was left at about 25–30° overnight. The reagent was used up. The mixture was diluted with an equal volume of water, then treated with 40 cc. of 10 per cent H_2SO_4 , and the MnO_2 was reduced with sulfur dioxide. The colorless crystals after collection with water gave a first fraction which weighed 2.85 gm. and melted at 218–221°. This fraction, which consisted essentially of α -oxodelphinine, was most readily recrystallized by solution in chloroform, addition of alcohol, and concentration to remove the chloroform. It formed flat needles which melted at 218–221°, depending on the rate of heating.

$$[\alpha]_D^{20} = -62^\circ \text{ (} c = 1.05 \text{ in acetic acid)}$$

$$[\alpha]_D^{20} = -55^\circ \text{ (} c = 0.275 \text{ in 95 per cent alcohol)}$$

Keller reported $[\alpha]_D^{20} = -52.2^\circ$ ($c = 0.42$ in alcohol).

Numerous analyses of this substance have given consistent results. Our more recent ones are reported.

$C_{33}H_{43}O_{10}N$.	Calculated.	C 64.56, H 7.07, OCH_3 20.22
$C_{32}H_{41}O_{10}N$.	"	" 64.07, " 6.89, " 20.69
	Found. (a)	" 64.72, " 6.91, " 19.88
	"	" 64.27, " 7.12
	" (b)	" 64.44, " 7.09
	" (c)	" 64.65, " 7.13

The mother liquor of the above first fraction which consisted of dilute acetone was further diluted and extracted with ether. The

aqueous phase on being rendered alkaline and extracted further with ether yielded a very small fraction of delphinine which had not been oxidized. The above ether extract of oxidation products was repeatedly washed with water. On concentration it gave readily a second fraction of needles (0.62 gm.) which melted at 218–221°. The mother liquor of this fraction on further concentration gradually deposited a mixture of needles and stout four-sided prisms. A suspicion of the presence of the latter had already been obtained during the manipulation of the first main fraction. The yield of this third fraction was 0.48 gm. and consisted essentially of β -oxodelphinine. This melted somewhat higher at 222–224° after preliminary softening. The mother liquor of this fraction on still further concentration and dilution gave a viscous mass which gradually crystallized to a mass of small, stout rhombs. After collection with a little alcohol 0.35 gm. of this fraction was obtained, which melted at 228–229° with decomposition and appeared to be practically pure β -oxodelphinine. It was recrystallized as in the case of the first substance by boiling down the chloroform solution with alcohol. The melting point remained at 228–229°.

$[\alpha]_D^{20} = +31^\circ$ ($c = 1.01$ in acetic acid)			
$C_{22}H_{43}O_{10}N$.	Calculated.	C 64.56,	H 7.07
	Found. (a)	" 64.47,	" 6.83
	" (b)	" 64.53,	" 6.85

Finally, from the last mother liquors a fifth and final fraction of similar crystals was obtained which amounted to 0.12 gm. The total yield of crystalline neutral material thus recovered from 5 gm. of delphinine was roughly 4.4 gm.

Hexahydro- α -Oxodelphinine—0.1 gm. of α -oxodelphinine was hydrogenated in acetic acid solution with 50 mg. of platinum oxide catalyst under 3 atmospheres pressure. The absorption of H_2 beyond the catalyst, 17 cc., was somewhat (about 1 mole) in excess of the 3 moles required, *viz.* 12 cc. The solution on concentration gave a resin which readily crystallized under ether. The collected material was recrystallized from ether and formed four-sided platelets which melted at 195°.

$C_{22}H_{49}O_{10}N$.	Calculated.	C 63.93,	H 7.97
$C_{22}H_{47}O_{10}N$.	"	" 63.43,	" 7.83
	Found.	" 63.74,	" 7.75
	"	" 63.77,	" 7.61

Pyro- α -Oxodelphinine—0.1 gm. of α -oxodelphinine was heated in an atmosphere of H_2 at 220° for 30 minutes. The resulting resin crystallized under ether. It was recrystallized from acetone and formed six-sided platelets which did not have a sharp melting point. The substance began to sinter above 150° and with gradual coloration slowly melted at 200 – 220° . It contained solvent. From methyl alcohol it formed flat needles which sintered to a resin above 238° and melted at 248 – 250° .

For analysis it was dried at 120° and 20 mm.

$C_{31}H_{39}O_8N$.	Calculated.	C 67.23, H 7.10
	Found. (a)	" 67.20, " 7.05
	" (b)	" 67.03, " 7.05

When pyro- α -oxodelphinine was heated in a sealed tube with 30 parts of 3 per cent methyl alcoholic hydrochloric acid, it formed a clear solution which on concentration yielded a sparingly soluble substance. The latter separated from methyl alcohol as minute wedge-shaped crystals which melted slowly at 280 – 284° after preliminary softening above 270° . It was apparently an isomer of the above substance.

$C_{31}H_{39}O_8N$.	Calculated.	C 67.23, H 7.10, $OCII_2$ 22.40
	Found.	" 66.72, " 7.03, " 21.84

Hexahydropyro- α -Oxodelphinine—Pyro- α -oxodelphinine was hydrogenated in acetic acid solution with platinum oxide catalyst. The apparent absorption of H_2 above the catalyst was excessive and between 4 and 5 moles. Recrystallized from a mixture of ether and petroleum ether the hydrogenation product formed needles which melted at 183 – 185° after preliminary sintering.

$C_{31}H_{46}O_8N$.	Calculated.	C 66.50, H 8.11
	Found.	" 66.48, " 8.22
	"	" 66.40, " 8.27

α -Oxodelphinine and Methyl Alcoholic Hydrochloric Acid—0.5 gm. of α -oxodelphinine was heated for 18 hours in a sealed tube at 100° with 10 cc. of 3 per cent HCl in absolute methyl alcohol. The resulting clear solution was chilled in dry ice before the tube was opened and then, after opening, the evolved gases were allowed to pass through barium hydroxide solution. Definite evidence of CO_2 cleavage was obtained. The clear reaction mixture was concentrated to dryness *in vacuo* and the resulting residue crys-

tallized readily from dilute alcohol. The first and second fractions amounted to about 0.2 gm. Recrystallized from a small volume of methyl alcohol, it separated as needles or long thin leaflets which on rapid heating melted at 220–222°. If the substance was first powdered, it sintered to a vitreous mass at this point and then melted at 235–236°. It appears that this behavior must be due to dimorphism, since the substance contained no appreciable amount of solvent of crystallization.

$C_{22}H_{43}O_9N$.	Calculated.	C 65.60, H 7.40, OCH_3 26.47
	Found.	" 65.32, " 7.38, " 24.62
	"	" 65.55, " 7.22
	"	" 65.25, " 7.14

14.64 mg. of substance were refluxed in a mixture of 3 cc. of alcohol and 3 cc. of 0.1 N NaOH for 4 hours and titrated back against phenolphthalein. Found, 0.27 cc.; calculated for 1 equivalent, 0.25 cc.

Under similar conditions 11.25 mg. of substance consumed 0.211 cc. of 0.1 N NaOH. Calculated for 1 equivalent, 0.192 cc.

When the above substance was melted in an H_2 atmosphere in a bath at 260° and allowed to cool, it crystallized. After recrystallization from dilute methyl alcohol it formed needles and leaflets which from melting point and mixed melting point proved to be unchanged starting material.

Found. C 65.73, H 7.17, OCH_3 25.65

The dilute alcoholic mother liquor of the above neutral methylated derivative was acidified with dilute HCl and extracted with ether. The latter yielded a very small additional amount of crystalline material which was not investigated further. When the remaining aqueous phase was rendered alkaline with excess dilute Na_2CO_3 and extracted with ether, an appreciable fraction was obtained on concentration which had basic properties. All attempts to crystallize this material were unsuccessful. On drying down, it formed a colorless, brittle, triboelectric, vitreous mass. Although the homogeneity of the substance is in doubt, it was analyzed after drying at 100° and 20 mm.

Found. C 66.29, H 7.68, OCH_3 23.95, $N(CH_3)$ 0.81

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THE ACONITE ALKALOIDS*

II. THE FORMULA OF OXONITINE

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Since the discovery by Carr (2) in 1912 of oxonitine, the neutral, sparingly soluble product of the oxidation of aconitine with permanganate and for which he proposed a formula $C_{23}H_{29}O_9N$, difference of opinion has prevailed as to the correct formulation of this substance. This interpretation assumed a new aspect when Späth and Galinovsky (3) in 1930 suggested the formula $C_{32}H_{43}O_{12}N$ on the basis not only of the C and H determinations but also on those of N, methoxyl, and benzoic acid. This formulation was supported by the more recent analyses of Lawson (4). However, a further slight modification of the formula to $C_{32}H_{41}O_{12}N$ was made by Majima and Tamura (5) on the basis of evidence reported by them, which appeared to be conclusive. Both aconitine, $C_{34}H_{47}O_{11}N$, and mesaconitine (6), $C_{33}H_{45}O_{11}N$, had been found to yield on oxidation the same oxonitine. Aconitine, on the one hand, has been shown to behave as if it contained an N-ethyl group (1, 5) by the formation of ethyl iodide in the N-alkyl determination and of ethylamine on fusion with KOH. On the other hand, mesaconitine (5) appears to contain an N-methyl group, since after saponification to mesaconine and subsequent demethylation (of OCH_3 groups) methylamine was the principal volatile base obtained on alkali treatment of the resulting demethylated mesaconine. Oxonitine, when similarly transformed to a demethylated oxonine, gave mainly ammonia and since it no longer shows an N-alkyl group in the alkyl determination Majima and Tamura conclude that the transformation of

* Earlier work on the aconite alkaloids was reported in 1936 (1).

aconitine and mesaconitine to oxonitine involves oxidative removal of the N-ethyl and N-methyl groups in these alkaloids with simultaneous oxidation of an adjoining CH_2 to CO group, with the formation of a lactam. The long known formation of acetaldehyde during the oxidation of aconitine to oxonitine and the detection by Majima and Tamura of formaldehyde among the oxidation products of mesaconitine seem to fit in with this interpretation. A precedent for the oxidative removal of an N-alkyl group is found in the conversion of tropine to tropigenine.

Thus a strong case appears to have been made for the nature of the transformation of these alkaloids into oxonitine. However, our recent experience may reopen this question. The analytical values which we have obtained with oxonitine prepared directly from Merck's crystalline aconitine as well as with aconitine purified over the hydrobromide according to the method of Majima and Sugimoto (7) have been consistently somewhat higher than those required by the formula $\text{C}_{32}\text{H}_{41}\text{O}_{12}\text{N}$ and in closer agreement with that of $\text{C}_{33}\text{H}_{43}\text{O}_{12}\text{N}$. This was supported by the analytical results obtained with the "isomer (?)"¹ previously described by us (1) and substantiated by our more recent work. At first we were reluctant to attribute too much significance to such analytical results until the experience with delphinine, as reported in the previous paper, had been encountered. Here from the analytical data the transformation of delphinine to oxodelphinine appears to proceed without loss of carbon atoms. Since this appeared to fit in with the oxonitine analyses, it was of importance to obtain other evidence. Accordingly, other derivatives of oxonitine were studied. On hydrogenation a *hexahydrooxonitine* was prepared. The analyses of this material supported a formula $\text{C}_{33}\text{H}_{49}\text{O}_{12}\text{N}$.

¹ Recent analyses of this more soluble oxidation product as well as those of its *hexahydro derivative* are in agreement with a formula $\text{C}_{34}\text{H}_{45}\text{O}_{12}\text{N}$ for the former. It must be formed from aconitine without loss of carbon atoms and is therefore an *oxoaconitine*. It is produced in larger amount than oxonitine. This at once brings up the question as to whether both oxonitine and oxoaconitine are simultaneously formed from aconitine itself by different reactions or whether the aconitine reported by different workers to yield oxonitine has not been homogeneous but contaminated by a C_{33} alkaloid which has been the source of oxonitine. This question is now under careful investigation.

Pyrooxonitine was then prepared according to Majima and Sugimoto (8). Contrary to the recent assumptions of Tamura (9), many analytical results which we have obtained with pyrooxonitine are in close agreement with a formula $C_{31}H_{39}O_{10}N$ rather than $C_{30}H_{37}O_{10}N$. Finally, on hydrogenation a crystalline *hexahydropyrooxonitine* was prepared, analysis of which supported the formula $C_{31}H_{45}O_{10}N$.

Thus from our experience the question of the exact relationship of oxonitine to aconitine has been reopened and can be finally settled only when the exact nature of the steps involved in the transformation of mesaconitine and aconitine to oxonitine has been determined.

In accordance with this, attempts at the degradation of oxonitine have been in progress. At this point we wish to report the results of a preliminary study of the cleavage of oxonitine with methyl alcoholic HCl. Roughly 40 to 50 per cent of a crystalline base has been obtained. Analyses indicate the presence of five methoxyl groups and at least one N-methyl group. Since CO_2 was formed during the production of the substance, the possibility appears that lactam cleavage occurs with loss of CO_2 and either with or without methylation of a resulting secondary amino group. Since in the case of the reaction of oxodelphinine with methyl alcoholic HCl the acetyl group is replaced by methyl, such a reaction may also occur here. The resulting base should then have a formula $C_{31}H_{45}O_{10}N$ or $C_{32}H_{47}O_{10}N$. However, this interpretation can be merely provisional and must await the results of further study. Attempts at further degradation (exhaustive methylation) of the substance are now in progress.

EXPERIMENTAL

Oxonitine—For the preparation of this substance we have used Merck's crystalline aconitine directly as such, and aconitine obtained from it by purification over the hydrobromide according to Majima and Sugimoto (7). In the latter case the recrystallized hydrobromide was reconverted to the base which was then recrystallized from methyl alcohol. This material then melted at 202–205°, depending on the rate of heating.

$C_{31}H_{47}O_{11}N$. Calculated, C 63.22, H 7.34; found, C 63.40, H 7.28

The purified alkaloid was oxidized with permanganate in acetone and acetic acid solution essentially according to the procedure of Barger and Field (10). After completion of the oxidation, the collected mixture of MnO_2 and oxonitine which had crystallized was treated in aqueous suspension with SO_2 to remove the MnO_2 . From 5 gm. of purified aconitine, 0.83 gm. of oxonitine was directly obtained. After recrystallization by addition of acetone to the solution in hot acetic acid, it separated as the usual heavy crystalline powder and melted at $279\text{--}282^\circ$ after preliminary softening.

$[\alpha]_D^{25} = -45^\circ$ ($c = 0.956$ in chloroform)			
$\text{C}_{32}\text{H}_{43}\text{O}_{12}\text{N}$.	Calculated.	C 61.36,	H 6.72
$\text{C}_{32}\text{H}_{41}\text{O}_{12}\text{N}$.	"	" 60.82,	" 6.55
	Found.	" 61.67,	" 6.61

Other preparations of equivalent character gave the following results.

Found.	C 61.39,	H 6.71
"	" 61.53,	" 6.63

When Merck's crystalline aconitine was directly employed without purification, similar results were obtained.

Found.	C 61.45,	H 6.65
"	" 61.24,	" 6.54
"	" 61.09,	" 6.70
"	" 61.00,	" 6.48

From 20 gm. of Merck's alkaloid, the yield of crude oxonitine was 5.5 gm. and 4.1 gm. of the following substance.

The acetone filtrate from the mixture of MnO_2 and oxonitine obtained above from the oxidation of recrystallized aconitine was concentrated to about 20 cc. On dilution with water an appreciable precipitate formed, which was collected with water. It proved to be a mixture. The filtrate from this after neutralization with sodium carbonate solution was extracted with chloroform. The washed chloroform extract was dried and concentrated to dryness. The residue when dissolved in a small volume of methyl alcohol slowly deposited prisms and rods. The yield of collected material was 1.3 gm. After recrystallization

from methyl alcohol it melted at 261° . The substance, like oxonitine, is not dissolved by dilute acid or alkali.

$$[\alpha]_D^{25} = -98^{\circ} (c = 0.956 \text{ in chloroform})$$

$C_{33}H_{43}O_{12}N$.	Calculated.	C 61.36,	H 6.72,	N 2.17,	OCH ₃ 19.22
	Found. (a)	" 61.40,	" 6.69,	" 2.57,	" 19.18
	" (b)	" 61.45,	" 6.75,	" 2.42,	" 19.07

From a run of 20 gm. of Merck's crystalline aconitine the yield of this substance was 4.1 gm. After recrystallization from methyl alcohol it melted at 261° .

$$[\alpha]_D^{25} = -99^{\circ} (c = 1.01 \text{ in chloroform})$$

	Found.	C 61.69,	H 6.51
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*Hexahydroaconitine*²—0.5 gm. of purified aconitine was dissolved in 5 cc. of alcohol with a few drops of HCl and hydrogenated with 50 mg. of platinum oxide catalyst under 3 atmospheres pressure. The absorption of H₂ was prompt and appeared to be complete in about 30 minutes. The absorption corresponded to 3 moles. The hydrogenated alkaloid as such was not obtained in crystalline form but was isolated as the beautifully crystalline perchlorate. This was obtained by addition of sodium perchlorate solution to the aqueous solution of the very soluble hydrochloride. Recrystallized by dilution of its concentrated solution in alcohol, it formed square-ended prisms which melted at $209\text{--}210^{\circ}$ after preliminary sintering.

$C_{33}H_{43}O_{11}N \cdot HClO_4$.	Calculated.	C 54.26,	H 7.24
	Found.	" 53.84,	" 7.35

On hydrolysis with water at $160\text{--}165^{\circ}$ hexahydrobenzoic acid was cleaved from it. After acidification with HCl and extraction with ether, this acid was obtained as an oil with characteristic odor. The concentrated HCl solution after addition of acetone crystallized on seeding with aconine hydrochloride. The collected material melted at $174\text{--}176^{\circ}$ and gave no depression with aconine hydrochloride.

Hexahydrooxonitine—0.15 gm. of oxonitine was suspended in

² Without isolation of the products, Freudenberg (11) demonstrated the absorption of 3 moles of H₂ by the benzoyl group in aconitine and oxonitine, and not in aconine.

acetic acid and shaken with 50 mg. of platinum oxide catalyst in hydrogen under 3 atmospheres pressure. Although absorption occurred promptly and all oxonitine dissolved within several hours, the operation was continued overnight. The filtrate from the catalyst left on concentration a crystalline residue. The substance formed prisms or minute rods from 95 per cent alcohol, which melted at 253° on rapid heating.

$C_{33}H_{46}O_{12}N$.	Calculated.	C 60.80, H 7.58
$C_{32}H_{47}O_{12}N$.	"	" 60.25, " 7.43
	Found.	" 60.74, " 7.49
	"	" 60.94, " 7.30

Pyrooxonitine.—This was prepared essentially according to Majima and Suginome (8). 0.4 gm. of oxonitine was heated in an atmosphere of H_2 at 280–285°. As soon as the substance melted, the tube was withdrawn from the bath. After cooling, the melt was dissolved in a few cc. of methyl alcohol and allowed to stand at 25° overnight. A small amount of unchanged oxonitine separated. The concentrated filtrate gave a resin which crystallized readily under chloroform. The collected material was recrystallized from chloroform and contained solvent. It was collected with chloroform and washed with a mixture of chloroform and petroleum ether.

For analysis it was dried at 120° and 20 mm.

$C_{31}H_{39}O_{10}N$.	Calculated.	C 63.55, H 6.72
$C_{30}H_{37}O_{10}N$.	"	" 63.01, " 6.53
	Found.	" 63.48, " 6.77
	"	" 63.68, " 6.90

In another experiment successive fractions were obtained from chloroform, which after recrystallization from chloroform gave the following figures.

Found.	(a)	C 63.30, H 6.45
"	(b)	" 63.34, " 6.85

Fraction (a) was then recrystallized from a mixture of alcohol and ether.

Found.	C 63.45, H 6.77
"	" 63.53, " 6.78

Majima and Suginome have reported a melting point of 231° for pyrooxonitine. In no case have we been able to duplicate this. Our substance as it separated from chloroform melted slowly to a

colorless resin at 160–170°. The material from alcohol-ether, which lost about 1 per cent in weight on drying, softened gradually to a resin at 170–180° which melted on further heating. Finally, when recrystallized from methyl alcohol the substance melted sharply at 180°. In the latter case a rotation was found of $[\alpha]_D^{25} = -127^\circ$ ($c = 1.14$ in methyl alcohol). This agrees closely with the values reported by Majima and Suginome, which approximated $[\alpha]_D = -128^\circ$ in methyl alcohol.

Hexahydropyroxonitine—0.15 gm. of pyrooxonitine was hydrogenated in alcoholic solution under 3 atmospheres pressure with 50 mg. of platinum oxide catalyst. After 2½ hours absorption had practically stopped. The absorption due to the substance was slightly in excess of 3 moles. The concentrated filtrate crystallized readily under ether. The collected material was recrystallized from acetone-ether and formed platelets which melted slowly at 160–163° to a viscous mass.

For analysis it was dried at 110° and 20 mm.

$C_{31}H_{46}O_{10}N$.	Calculated.	C 62.91, H 7.67
$C_{30}H_{45}O_{10}N$.	"	" 62.35, " 7.51
	Found.	" 62.81, " 7.67
	"	" 62.76, " 7.53

Base, from Oxonitine—50 mg. of oxonitine were heated in a tube with 2 cc. of a 6 per cent solution of HCl in dry methyl alcohol at 100° for 18 hours. A clear solution resulted which was concentrated *in vacuo* to dryness. The resinous residue was dissolved in water and after being made alkaline with dilute Na_2CO_3 the mixture was extracted with chloroform. The dried extract on concentration gave a residue which crystallized as platelets from ethyl acetate. The yield was 25 mg. The substance proved to be a base and dissolved readily in dilute acid. It melted at 250° after preliminary sintering. It was easily soluble in alcohol, acetone, less readily in ethyl acetate, and but sparingly soluble in ether.

$C_{31}H_{46}O_{10}N$.	Calculated.	C 62.91, H 7.67, N 2.37
$C_{32}H_{47}O_{10}N$.	"	" 63.43, " 7.83, " 2.31
	Found.	" 62.49, " 7.58
	"	" 62.97, " 7.61, " 2.61
$C_{31}H_{46}O_{10}N$.	Calculated.	5(OCH ₃) 26.22, N(CH ₃) 2.54
$C_{32}H_{47}O_{10}N$.	"	" 25.62, " 2.49
	Found.	" 24.94, " 3.19

In another experiment in which 1 gm. of oxonitine was heated with 25 cc. of 4.7 per cent methyl alcoholic HCl, the tube was chilled to low temperature before opening. On opening, it was at once equipped with a tube leading into Ba(OH)₂ solution. As the tube warmed up, the presence of CO₂ in the evolved gases was readily noted. No attempt at a quantitative estimation was made. In this experiment the yield of base was 0.36 gm.

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THE ERGOT ALKALOIDS

XVII. THE DIMETHYLINDOLE FROM DIHYDROLYSERGIC ACID

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Among the volatile products formed on fusion of dihydrolysergic acid (1) an indole fraction was obtained from which a picrate was isolated in very small amount which melted at 148-150°. The analytical figures obtained with it approximated those of the picrate of an indole $C_{11}H_{13}N$. The indole still gave the dimethylaminobenzaldehyde test and therefore presumably had the α (or β) position still free (2). It was not identical with a β -*n*-propylindole. Since it was formed in such small amounts and was very difficult to purify, we have turned again more recently to the previously reported indole acid contained in the non-volatile alkali melt, which was also formed in very small amount (1).

This indole acid fraction was again prepared and subjected to low pressure distillation during which decarboxylation occurred. The resulting indole after recrystallization was finally obtained in very small yield with a melting point of 115-117°. A picrate prepared from one of the fractions melted at 185-187°. Since there was not sufficient of either the indole itself or its picrate with these melting points for analysis, analyses were obtained with several picrate fractions of somewhat lower melting points (175-183° and 164-168°). These analyses were in fair agreement with the figures required by a dimethylindole. Since the indole still gave the dimethylaminobenzaldehyde reaction, it appeared probable that it could be a 3,4-dimethylindole resulting by decarboxylation of a 4-methylindole-3-acetic acid. This would be expected as a scission product from the structure which we have derived for lysergic acid.

As a check on this conclusion the indoles were prepared from

m-tolylhydrazine and propionaldehyde. Mendlik and Wibaut (3) had previously attempted the same synthesis and obtained but one substance (m.p. 116–117° and picrate m.p. 179–180°) which they concluded to be 3,6-dimethylindole. However, we have obtained two isomers, as would be expected,¹ one which must be 3,4-dimethylindole and the other 3,6-dimethylindole. The identity of these has not been directly established except by inference from the comparison with the above indole from lysergic acid. One of these, which melted at 117–118°, gave a picrate melting at 182–183° and was thus identical with that described by Mendlik and Wibaut. Neither this indole nor its picrate gave a depression with the substances obtained from lysergic acid and agreed in other properties with these substances. It may therefore be inferred that this substance is 3,4-dimethylindole and not the 3,6 derivative. The formation of a 3,6 derivative, barring unlikely rearrangements, is incompatible with the structure that we have derived for lysergic acid and which is at the same time a derivative of naphthalene, quinoline, and indole. The second synthetic indole which melted at 90–93° and gave a picrate melting at 163–164° would therefore be 3,6-dimethylindole.

The attempt will be made, when opportunity presents, to confirm this by the synthesis of 3,4-dimethylindole by another method.

EXPERIMENTAL

3,4- and 3,6-Dimethylindoles—5 gm. of propionaldehyde were treated with 10.4 gm. of *m*-tolylhydrazine and warmed. The hydrazone was extracted with ether and the solution was dried over K₂CO₃. After concentration the residue was treated with 30 gm. of anhydrous zinc chloride and the mixture was heated in an oil bath raised to 165°. When the reaction was completed, the contents of the flask were washed out with ether and excess 10 per cent HCl. The washed and dried ether extract was concentrated in an apparatus for sublimation of the residue. All the material subliming up to an oil bath temperature of 125° and under 0.2 mm. pressure was collected and the residue discarded. The sublimate which weighed about 3 gm. was dissolved in 30 cc. of

¹ The formation of two isomeric oxindoles from the *m*-tolylhydrazide of propionic acid has already been reported (4).

warm petroleum ether. When chilled to -15° , crystallization began. The crystals were filtered off without washing and the filtrate was set aside to be treated as described below.

The crystalline fraction after repeated crystallization from petroleum ether formed flat needles which melted at $117-118^{\circ}$.

$C_{10}H_{11}N$. Calculated, C 82.76, H 7.64; found, C 82.89, H 7.69

This indole yielded a picrate from ethyl alcohol forming dark red needles which melted at $182-183^{\circ}$.

$C_{18}H_{14}O_7N_4$. Calculated, C 51.34, H 3.74; found, C 51.37, H 3.78

The above petroleum ether filtrate from the crystalline indole was concentrated and transferred to a 22 cm. microfractionating column. Fractionation was carried out under approximately 0.2 mm. pressure. Five fractions were collected. Each fraction was about 0.15 gm. with the exception of the second which weighed 0.33 gm.

The second fraction completely solidified on the condenser and showed a melting point of $60-90^{\circ}$. When dissolved in 3 cc. of petroleum ether and chilled to -15° , crystallization began. 0.28 gm. of broad, thin leaflets was collected, which melted at $90-93^{\circ}$

0.1 gm. was converted to the picrate in 3 cc. of ethyl alcohol. 0.175 gm. of needles was collected which melted at $163-164^{\circ}$.

$C_{18}H_{14}O_7N_4$. Calculated, C 51.34, H 3.74; found, C 51.45, H 3.97

The indole recovered from the picrate after redistillation melted at $90-92^{\circ}$.

$C_{10}H_{11}N$. Calculated, C 82.76, H 7.64; found, C 82.97, H 7.65

Dimethylindole from Dihydrolysergic Acid—1.2 gm. of dihydrolysergic acid were ground with 7 gm. of KOH and placed in a fusion apparatus as previously described (1). The salt bath for heating the mixture was held at 300° for 0.5 hour.

The volatile material from the reaction was worked up in the manner previously given and 10 mg. of the indole fraction were obtained. Since this proved difficult to purify further, attention was turned to the alkali melt.

This melt was dissolved in water and extracted with ether. The latter was discarded. The aqueous layer was then acidified to

Congo red with HCl and extracted repeatedly with ether. This extract was concentrated in a molecular still for distillation of the residue. 135 mg. of distillate were collected up to an oil bath temperature of 200° and under a pressure less than 0.001 mm. The distillate was dissolved in ether and extracted with NaOH solution. The alkaline extract was then acidified with HCl. The acid material which separated was reextracted with ether. Since it could not be directly crystallized, it was redistilled in a microstill under 0.5 mm. pressure with the temperature of the oil bath raised to 250°. During this procedure, the production of an indole by decarboxylation was obvious from the odor of the distillate. 87 mg. of material were collected. This was dissolved in ether and shaken out with NaOH solution to remove undecomposed indole acids. The ether layer upon evaporation gave an oily indole fraction which was redistilled in a microstill. 7 mg. distilled up to 150° under 0.3 mm. pressure. The product melted at 80–100°. Upon recrystallization from 0.02 cc. of petroleum ether, 3 mg. of flat needles were obtained which melted at 108–112°.

When this was treated with an equivalent of picric acid in 0.15 cc. of ethyl alcohol, 3.2 mg. of red needles were obtained which melted at 182–186°. After recrystallization from alcohol the substance melted at 185–187°. A mixed melting point with the higher melting synthetic picrate obtained above showed no depression.

The final picrate was recombined with its mother liquor and evaporated to dryness. The residue (3.2 mg.) was treated with a small volume of sodium hydroxide and a few drops of ether. The ether layer was washed with NaOH solution until all color of picric acid had disappeared and then evaporated to dryness in a microstill for redistillation of the indole. 1.5 mg. distilled up to 150° and under 0.3 mm. This material melted at 115–116°. After recrystallization from 0.01 cc. of petroleum ether, 0.8 mg. of long flat needles was obtained which melted at 115–117°. A mixed melting point with the higher melting synthetic indole reported above showed no depression.

Since the supply of the pure, high melting indole from dihydrolysergic acid was exhausted in melting point determinations, there was not sufficient for analysis as the picrate or the indole itself.

Analytical data were obtained on a lower melting picrate (164–168°) obtained in a preliminary run as follows:

$C_{18}H_{14}O_7N_4$. Calculated, C 51.34, H 3.74; found, C 51.70, H 3.92

From the mother liquors of the purified indole, 2.2 mg. of indole were recovered which melted at 80–105°. A picrate (3 mg.) prepared from this melted at 175–183°. This was not recrystallized, since there was not enough for both recrystallization and analysis. The analysis gave C 51.90, H 4.06.

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THE VERATRINE ALKALOIDS

V. THE SELENIUM DEHYDROGENATION OF CEVINE

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Blount (1) and Blount and Crowfoot (2) have described the isolation and x-ray crystallographic study of several products formed on the selenium dehydrogenation of cevine. The principal substance was an apparently tetracyclic base, cevanthridine, to which a formula $C_{23}H_{25}N$ was assigned, and in smaller amount a tricyclic phenol, $C_{17}H_{16}O$, cevanthrol, measurements of which suggested a substituted phenanthrol, and finally in very small amount a hydrocarbon of questionable homogeneity. The correct interpretation of these substances will doubtless be important in the final derivation of the ring system of cevine. Since the study of the pyrolysis of cevine with zinc dust and soda lime had led us (3) to a series of simpler pyridine and piperidine derivatives, it appeared strange that none of these was encountered among the products of the selenium dehydrogenation. Several years have passed since the last paper of Blount and Crowfoot on this subject, and we have accordingly made a supplementary study from this standpoint. As a matter of fact, it has been found possible to isolate other substances from among the products of the dehydrogenation of cevine while confirming the production of cevanthridine and cevanthrol. In addition, we have collected the more volatile products formed during the dehydrogenation. In this material but an inappreciable amount of unstable acid or neutral fraction was found. However, on fractionation of the basic fraction β -picoline and the 2-ethyl-5-methylpyridine previously encountered on zinc dust distillation were obtained. In addition, a higher boiling (about 216°) oxygen-containing base was isolated,

the analysis of which approximated a formula C_8H_9ON which was supported by analysis of its picrate.

The mixture of dehydrogenation products which remained in the selenium melt was extracted with ether. The material so obtained was chromatographically fractionated, as shown in the experimental part. In addition to cevanthridine and cevanthrol which agreed essentially in properties with those recorded by Blount and Crowfoot, a base was obtained which melted at 229° but which gave analytical figures difficult to interpret. However a formula $C_{25}H_{25}N$ is a possibility.

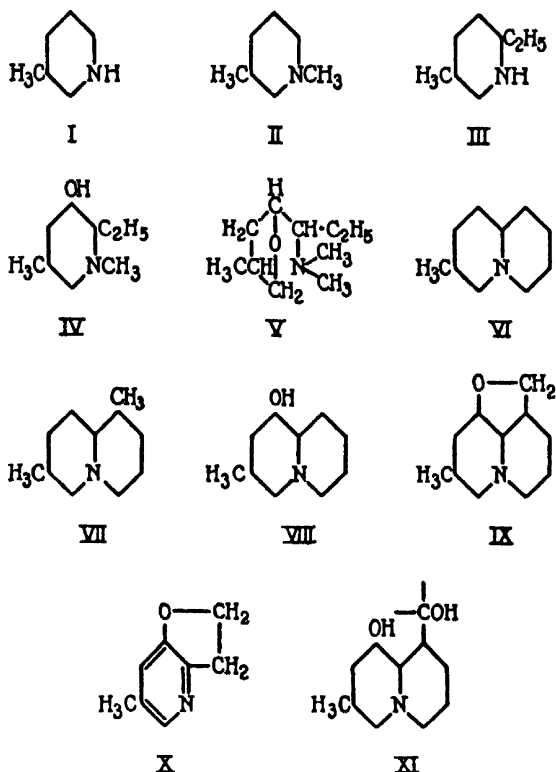
A third base which melted at 186° gave figures which approximated those of a formula $C_{24}H_{25}N$.

From hydrocarbon fractions of the chromatograph, two hydrocarbons were obtained which approximated homogeneity. One of these, which melted at $138-144^\circ$, gave on analysis figures close to those required for a hydrocarbon, $C_{17}H_{18}$, and therefore possibly corresponding to cevanthrol, $C_{17}H_{16}O$. Blount reported a very small yield of a hydrocarbon also melting at $139-143^\circ$ but admittedly impure, since the analytical figures were obviously inconclusive; *viz.*, C 90.3, H 8.0. Analysis of our second hydrocarbon which melted at $116-118^\circ$ agreed with a formula $C_{18}H_{18}$ and it is therefore apparently a homologue of the preceding hydrocarbon. Further investigation of these substances, both bases and hydrocarbons, which are obtained in poor yield is now in progress.

In recapitulation, among the bases which have been isolated as degradation products of cevine may be mentioned β -picoline, partly racemized *d*- β -pipecoline (I), *d*-N-methyl- β -pipecoline (II), 2-ethyl-5-methylpyridine, a homologue $C_9H_{13}N$, 2-ethyl-5-methylpiperidine (III), the dicyclic base $C_{10}H_{19}N$ (methyloctahydropyridocoline ?) (VI), and a homologue (?) $C_{11}H_{21}N$ (VII), a possible hydroxymethyloctahydropyridocoline $C_{10}H_{19}ON$ (VIII), and a crystalline, possibly tricyclic base $C_{11}H_{19}ON$ (IX). In the degradation of the betaine, $C_{28}H_{45}O_8N$, (the so called des-base) (4) from cevine methiodide, a major product proved to be an N-methyl hydroxy base, $C_9H_{19}ON$, probably an N-methyl-2-ethyl-5-methylhydroxypiperidine (IV).

When the attempt was made, as already described (4), to carry this base through the steps of exhaustive methylation, its

methiodide did not yield an expected unsaturated des-base but apparently an oxido compound, which was isolated as its methiodide. This dimethylamino oxido base would then have formula



(V). Finally may be mentioned the oxygen-containing base C_8H_9ON just isolated from the selenium reaction (X) (?).

If certain assumptions are made which are at present admittedly speculative but appear to be justified and which must, of course, be proved by further work, all of these bases may be correlated—as is apparent from the structural formulas given. From such degradation products a partial structure for cevine would be suggested as given in (XI), to which other rings must be in part directly fused or attached by a side chain.

EXPERIMENTAL

Selenium Dehydrogenation of Cevine

Varying amounts of cevine were ground with twice their weight of selenium powder and placed in a round bottom flask of 5 or 6

times the volume of the mixture. The neck of the flask was lengthened by sealing on a piece of tubing about 50 cm. in length. A slow stream of hydrogen was passed through a glass tube which extended through the top of the flask and under the melt in the bottom. An exit tube was sealed on the flask about 5 cm. above the bulb and led to a flask immersed in ice. This served as a condenser for the volatile material emerging from the reaction. The flask was heated by a salt bath which just covered its bulb.

Volatile reaction products began to distil from the mixture at a temperature of 280° and continued as the temperature was raised to 320°. After 1 hour at this temperature little additional distillate was formed. In our experience long heating at this temperature alters somewhat the character of the material remaining in the melt. Better yields of soluble material are obtained with shorter heating, but the products are more difficult to separate.

Volatile Degradation Products—The volatile products from a 10 gm. run were collected and acidified to Congo red with HCl. The acid solution was then shaken out with a small volume of ether. The ether solution was fractionated through a column. It appeared to contain only about 50 mg. of a very unstable oil which was not examined further. The acid aqueous layer was made strongly alkaline with solid KOH and extracted with a small volume of ether. The ether was dried over K_2CO_3 and carefully concentrated. The residue was fractionated in a microstill. After a small amount of residual ether had distilled off, about 450 mg. of an oil which distilled up to a bath temperature of 115° at 0.5 mm. pressure were collected. This was then refractionated carefully in a microfractionating column 10 cm. in length. The following fractions were obtained with the bath temperature at 90–105° and with pressure diminishing from 30 mm. to 0.5 mm., as recorded in Table I.

Fraction 1 gave a boiling point and analytical figures suggesting β -picoline: calculated for C_6H_7N , C 77.37, H 7.58. It was possibly contaminated with water, since suitable precautions for drying had not been taken. It was definitely identified as the picrate. 10 mg. of the oil were treated with an equivalent of picric acid and the resulting picrate was crystallized from ethyl alcohol. 18 mg. were collected which melted at 145° and showed

no depression in mixed melting point with synthetic β -picoline picrate.

$C_8H_7N \cdot C_6H_3O_7N_3$. Calculated, C 44.70, H 3.13; found, C 44.76, H 3.16

Fraction 4 gave a boiling point and analytical figures agreeing with 2-ethyl-5-methylpyridine: calculated for $C_8H_{11}N$, C 79.27, H 9.15. 20 mg. of the oil when treated with an equivalent of picric acid in ethyl alcohol gave 40 mg. of picrate which melted at 142° and gave no depression when mixed with 2-ethyl-5-methylpyridine picrate obtained from the zinc dust distillation of cevine (3).

$C_8H_{11}N \cdot C_6H_3O_7N_3$. Calculated, C 47.98, H 4.03; found, C 47.97, H 3.89

TABLE I
Volatile Degradation Products of Cevine

Fraction No.	Weight	Bath temperature	Column temperature	Pressure	Micro b.p. at 760 mm.	Analysis	
						C	H
	mg.	$^\circ C.$	$^\circ C.$	mm.	$^\circ C.$		
1	60	93	82	30	143	76.13	7.84
2	60	96	85	20	162	77.22	8.65
3	60	96	87	17	173	78.97	9.08
4	60	96	87	9	175	79.21	9.01
5	60	96	87	3	187	75.84	8.85
6	60	105	90	0.5	216	71.50	7.24
7	20	Residues					

Fraction 5 showed a lower carbon content which indicated the presence of oxygen. Fraction 6 showed a still lower carbon content and gave analytical figures which agreed roughly with a base C_8H_9ON .

C_8H_9ON . Calculated, C 71.08, H 6.71; found, C 71.50, H 7.24

Analysis of the picrate supported this formula. 20 mg. of the oil treated with an equivalent of picric acid in acetone gave 22 mg. of crystalline material which melted at 150 – 151° .

$C_8H_9ON \cdot C_6H_3O_7N_3$. Calculated, C 46.14, H 3.30; found, C 46.22, H 2.98

Non-Volatile Degradation Products—The selenium-containing non-volatile residue from the dehydrogenation of 20 gm. of cevine was ground and extracted overnight with ether in a Soxhlet

apparatus. The ether extract was filtered from a small amount of selenium and evaporated to dryness. 5.6 gm. of material remained. The residue was dissolved in benzene and evaporated to dryness again to remove all ether. It was then dissolved in 100 cc. of benzene and run through a chromatograph prepared with Brockmann's aluminum oxide (from E. Merck, Darmstadt). The chromatograph contained 350 gm. of aluminum oxide and was 8 feet in length. Material began to come through after 310 cc. of solvent had drained. Succeeding volumes were then collected and considered as fractions.

All of the fractions were separately treated with small volumes of acetone. Fractions 1 to 3 showed no tendency to crystallize,

TABLE II
Less Volatile Degradation Products of Cevine

Fraction No.	Volume of solvent	Weight of material eluted	Weight of crystalline material from acetone	Weight of neutral fraction
	cc.	mg.	mg.	mg.
1	120	370	0	400
2	40	200	0	
3	40	340	0	30
4	40	315	25	0
5	40	290	65	0
6	40	240	95	0
7	50	230	100	0
8	50	190	65	0
9	400	430	95	0

while all of the succeeding fractions gave crystalline material which was collected on a special centrifuge filter. The amount of such material is shown for each fraction in Table II. The crystalline material from Fractions 4 to 8 did not melt sharply but gave analytical results indicating the cevanthridine reported by Blount (1). Careful examination by fractional crystallization of each of these fractions seemed to indicate that they were the same and they were finally combined. Upon recrystallization twice from ethyl alcohol, broad thin leaves were obtained which melted at 211–212° (corrected). Blount reported a melting point of 207° for his substance.

$C_{23}H_{25}N$. Calculated, C 87.56, H 7.99; found, C 87.56, H 7.90

Material was obtained from the mother liquors by fractional crystallization. Although such material gave considerably lower melting points, the analytical figures did not differ appreciably from those of cevanthridine. With the amount of material available it was not possible to effect a separation. It is possible that the mixture consists of cevanthridine and isomeric or homologous substances which have similar properties.

The crystalline material from Fraction 9 showed a higher carbon and a lower hydrogen content; found, C 88.30, H 7.28. After recrystallization from acetone and twice from benzene, broad flat needles were obtained which melted at 229–230° (corrected). The analytical data were not entirely satisfactory but were perhaps in best agreement with a formula, $C_{25}H_{25}N$. This point is still under investigation.

$C_{25}H_{25}N$. Calculated.	C 88.44, H 7.43, N 4.12
Found.	" 88.34, " 6.83, " 3.97

Fractions 1 to 3 were separately dissolved in ether and shaken with a small volume of 10 per cent HCl. In each case an insoluble hydrochloride separated. The resulting mixture was centrifuged and the supernatant ether layer was removed and dried over K_2CO_3 . The ether extract from Fraction 3 gave little residue, while in the cases of Fractions 1 and 2 material was obtained which analysis indicated to be of hydrocarbon nature. This material from the two fractions was combined. The amount was 400 mg. Its further investigation will be described below.

In the case of Fractions 4 to 9 the mother liquors obtained from the cevanthridine, etc., crystallizations were similarly treated with HCl and ether. Although hydrochlorides were obtained as described below, none of these fractions yielded a hydrocarbon fraction.

In the case of each of the fractions the suspension of the hydrochlorides was readily extracted with chloroform. The chloroform extracts from Fractions 4 to 9 were combined and dried over calcium chloride. After removal of the chloroform, the residue was crystallized from a small volume of acetone. 230 mg. of material were thus obtained. It was recrystallized by dissolving in a small volume of chloroform with addition of acetone. 180

mg. were obtained which melted at 188–190°. Analysis indicated loss of HCl on drying.

A solution of the salt in chloroform was shaken with sodium hydroxide solution and dried over K_2CO_3 . After removal of the chloroform the residue was recrystallized twice from acetone. The base melted sharply at 186° (corrected). It gave analytical figures indicating a formula of $C_{24}H_{25}N$ or $C_{23}H_{23}N$.

$C_{24}H_{25}N$.	Calculated.	C 88.02, H 7.70, N 4.28
$C_{23}H_{23}N$.	"	" 87.98, " 7.40, " 4.47
	Found.	" 88.12, " 7.40, " 4.11

The hydrocarbon fraction from Fractions 1 and 2 was placed in a small sublimation apparatus under a pressure of 0.3 mm. 300 mg. of material sublimed up to a bath temperature of 220°. This was combined with a similar fraction from another run and placed in a microfractionating column 22 cm. in length. A pressure of approximately 0.01 mm. was maintained at the mouth of the apparatus. Four fractions were taken, each weighing about 100 mg. The first fraction did not crystallize and smelled strongly of selenium compounds. The ensuing three fractions crystallized mainly at room temperature. A residue of about 250 mg. remained in the still and could not be distilled up to a temperature of 220°. The second fraction of these yielded 10 mg. of crystalline material from methyl alcohol which melted at 138–144° with previous sintering. When a micromelting point was taken, this was found to be 138–150°. The substance gave analytical data approximating those required by the hydrocarbon, $C_{17}H_{16}$, derived from cevanthrol by removal of oxygen.

$C_{17}H_{16}$. Calculated, C 92.67, H 7.32; found, C 92.68, H 7.46

The third fraction gave 25 mg. of crystalline material from methyl alcohol, which showed a micromelting point of 104–108°. Upon recrystallization, 17 mg. of substance were obtained (111–114°). Another recrystallization yielded 8 mg. of thin leaflets which gave a micromelting point of 116–118°. The analytical data agreed with the figures of a homologue of the hydrocarbon, $C_{17}H_{16}$.

$C_{18}H_{18}$. Calculated, C 92.24, H 7.77; found, C 92.06, H 7.71

The fourth fraction upon recrystallization from methyl alcohol yielded 15 mg. of material with a micromelting point of approximately 120°, with some of the crystals remaining unmelted until 150°. Found, C 92.51, H 7.66.

The column was then eluted with ether. Upon evaporation 550 mg. of substance were obtained from which we have not as yet obtained crystalline material.

The column was then eluted with acetone. The eluate gave about 300 mg. of residue upon evaporation of the acetone. This likewise has not as yet yielded crystalline material.

Elution was then made with methyl alcohol. On evaporation of the solvent 400 mg. of a residue were obtained which was placed in a molecular still under a pressure that was less than 0.1 μ . 280 mg. distilled up to a bath temperature of 230°. This readily crystallized under benzene and after collection amounted to 100 mg. On two recrystallizations from benzene, 40 mg. resulted which corresponded to the cevanthrol isolated by Blount and Crowfoot (2). It melted at 195–196° (corrected). Blount and Crowfoot reported a melting point of 197–198° for the substance.

$C_{17}H_{16}O$. Calculated, C 86.39, H 6.83; found, C 86.40, H 6.63

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THE VERATRINE ALKALOIDS

VI. THE OXIDATION OF CEVINE

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In previous work on the degradation of cevine, high temperature pyrolytic procedures (soda lime distillation or selenium dehydrogenation) have been rewarding in the search for degradation products. These, however, have been limited in number and their nature has made them difficult to relate to the parent substance with the exception of those derived from its basic portion. More recently it has been possible, for the first time, to achieve a crystalline oxidation product. Chromic acid in dilute sulfuric acid has given a mixture from which an acid fraction in good yield has been separated. This fraction, still a mixture, could not be directly crystallized. However, when heated to 180° evolution of carbon dioxide occurred, with production in good yield of a crystalline product which was non-nitrogenous (m. p. 273-278°), $[\alpha]_D^{25} + 47.6^\circ$ ($c = 0.925$ in pyridine). Analysis indicated a formula $C_{14}H_{14}O_6$. Calculated: C, 60.41; H, 5.07. Found: C, 60.51; H, 5.20.

Diazomethane gave a product which crystallized readily from acetone (m. p. 165-166°). Analysis indicated 2 methoxyl groups. Calcd. for $C_{16}H_{18}O_6$: C, 62.75; H, 5.92; OCH_3 , 20.26. Found: C, 62.96; H, 6.07; OCH_3 , 19.90. The molecular weight by the Rast method was found to be 326. Calculated for $C_{16}H_{18}O_6$, 306.14.

15.06 mg. of the acid in 2.5 cc. of alcohol was titrated against phenolphthalein. Found 1.014 cc. of 0.1 *N* NaOH; calculated for 2 equivalents, 1.082 cc. No additional alkali was consumed after boiling with excess 0.1 *N* sodium hydroxide.

14.96 mg. of the ester required 0.478 cc. of 0.1 *N* NaOH for direct titration in the cold. Calculated for one equivalent: 0.488 cc. After boiling for two hours an additional 0.437 cc. of alkali was consumed.

From these data it appears likely that the substance contained a labile lactone group and that one of the two methyl groups introduced with diazomethane involved a phenolic or enolic hydroxyl. Presence of a phenolic or enolic group was indicated by a prompt, deep red-purple color obtained with ferric chloride. The acid as well as the ester coupled with diazotized sulfanilic acid. On hydrogenation with platinum approximately 3 moles of hydrogen was absorbed, but the product could not be crystallized. It no longer gave the original color reactions.

These properties suggest that the substance contained a benzene ring and is possibly the lactone of a substituted tetrahydronaphthalene. However, the substance when treated with alkali gave a solution from which carbon dioxide was liberated on acidification. From this solution crystalline degradation products could be isolated which suggested that the original acid is capable of undergoing decomposition similar to that of β -ketonic acids. The phenolic or enolic character may therefore be due to such a grouping and thus the unsaturation of the substance to the presence of ketonic linkages rather than to a benzene ring. The details of this further degradation must be left to a later communication.

Our previous studies [*J. Biol. Chem.*, **119**, 141 (1937); **120**, 447 (1937)] have indicated that the basic portion of the cevine molecule is a substituted octahydropyridocholine. The substance, $C_{14}H_{14}O_6$, must be derived from that portion of the molecule all or in part distinct from the basic portion.

THE FREE RADICALS OF THE TYPE OF WURSTER'S SALTS

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The univalent oxidation products of the aromatic *p*-diamines, or Wurster's salts,¹ are free radicals which may polymerize in a sufficiently concentrated solution and at low temperature or in the solid state.² This paper is concerned with their properties, especially their stability and color, in dilute solution where they appear only as free radicals. They are in general much more stable than the corresponding quinone diimines although the latter are not radicals. The absorption spectra of these radicals show a very distinct pattern of bands in the visible range of wave lengths varying according to substitutions of the hydrogen atoms either at the amino groups or at the ring. The influence of substitutions at the amino groups has been described by Piccard. This study will be extended here, and a study of the effect of substitutions at the ring will be added.

1. *Stability*.—The study of stability in the dissolved state is complicated by the fact that it is impossible to obtain any pure solution of these radicals at all. They exist only in equilibrium with one molecular species at a lower level of oxidation, the diamine, and another at a higher level of oxidation, the diimine. Since the latter are very unstable compounds, liable to undergo irreversible changes, such a system may undergo irreversible changes, which are not directly due to any lability of the radical itself. For this reason the criteria for stability or lability of the radical should be discussed first. Stability in general may be conceived as the reluctance to undergo chemical reactions. One is faced with an obvious ambiguity with respect to the definition and measurement of stability, because the

(1) R. Willstätter and J. Piccard, *Ber.*, **41**, 1458 (1908); J. Piccard, *ibid.*, **46**, 1843 (1913); E. Weitz, *Z. Elektrochem.*, **34**, 538 (1928); L. Michaelis, *THIS JOURNAL*, **53**, 2953 (1931); L. Michaelis and E. S. Hill, *ibid.*, **55**, 1487 (1933).

(2) J. Piccard, *Ann.*, **381**, 351 (1911); *Ber.*, **59**, 1438 (1926).

tendency to enter into a chemical reaction depends also on the nature of the substance with which the substance in question is to react. This is true for all reactions except those which are strictly monomolecular such as radioactive decompositions. Among the various chemical reactions which a radical is inclined to undergo there are three types which might be chosen as criteria for stability. If we designate, as previously, the three levels of oxidation-reduction by R, S, and T (the diamine, the semiquinone radical, and the diimine), and the dimerized radical as D, the stability of the radical may be measured either by the equilibrium constant of the reversible reaction



or by the equilibrium constant of the reversible reaction



or (3) by the rate at which the free radical undergoes any irreversible reaction. The kind and rate of such a reaction depends on the solvent and on the nature of the substance with which the radical is to react.

The study of each such reaction will lead to a different notion of the stability of the radical. If we choose the equilibrium constant of reaction (1) as criterion for stability and compare the stabilities of various radicals of the homologous series, not only S is varied but also R and T. If we choose the constant of reaction (2), not only S is varied but also D. So the best comparison available of stabilities of the radicals among themselves seems to be a reaction of the type (3) as standard method, chosen in such a way that only the radical is varied, and the substance with which it reacts is always the same, namely, nothing else but a properly chosen solvent. Thus it seems to be the fairest and most natural method for measuring the stability of the radical just to measure its lifetime in a properly chosen solvent. This criterion of stability is preferable furthermore because the equilibrium constant of reaction (1) cannot be measured (except for diamino-durene) on account of the lability of the quinone diimine, and the process (2) from left to right does not take place to any measurable degree in dilute solution.

The criterion for stability, then, will be the lifetime of the radical

in a properly chosen solvent. There is only one difficulty involved in this procedure which must be first overcome. It is characteristic of the systems to be investigated in this paper that the free radicals (the S forms, Wurster's salts in the present case) are much more resistant to irreversible changes when exposed to a solvent, than are the T forms, the quinone-diimines, though the latter are not free radicals. It is known that quinone diimines in all solvents not entirely water free, are very unstable; whereas some of the free radicals such as Wurster's blue, are very stable compounds even in an aqueous solution. Consequently, if in some particular case a radical in a certain solution has only a short lifetime, it cannot be decided directly whether the irreversible disappearance of the radical is due to its own lability, or to the lability of the diimine with which it is in equilibrium. How much of the labile diimine is present in equilibrium with the radical depends on the thermodynamical constant of the equilibrium $R + T \rightleftharpoons 2S$. This constant need not show any simple relation to the tendency of the radical to undergo irreversible reactions. Hence we have to find a method to distinguish what may be designated as the *direct*, and the apparent or *indirect*, lability of the radical. The aim is to measure the direct lability of the radical. The lifetime of the radical in a properly chosen solvent will be taken as a measure of its stability provided the lability of the radical under the experimental conditions is a direct one, and not an indirect one. In order to show how this idea can be used in practice we shall describe four typical cases.

First Case.—If a solution of tetramethyl-*p*-phenylenediamine bi-hydrochloride, about 0.01 *M*, in an aqueous acetate buffer, *pH* 4.6, is oxidized with a very small amount of bromine, say not more than 1 atom of bromine to 50 or 100 molecules of the diamine, the intense blue color of the radical developed is stable for practically any length of time. It is known that the quaternary diimonium compound derived from this diamine is a compound extremely unstable in water. According to Willstätter even in the solid state it readily decomposes with production of formaldehyde. This experiment shows that the amount of the diimonium compound formed under the conditions chosen in equilibrium with the blue radical is vanishingly small, and the radical itself is very stable.

Second Case.—When the same experiment is performed with *p*-phenylenediamine instead of its tetramethylated derivative, the result is quite different. When aqueous acetate buffer is chosen as a solvent, the yellow color of this radical primarily formed on addition of bromine, turns pink in a few minutes. The rate at which this irreversible

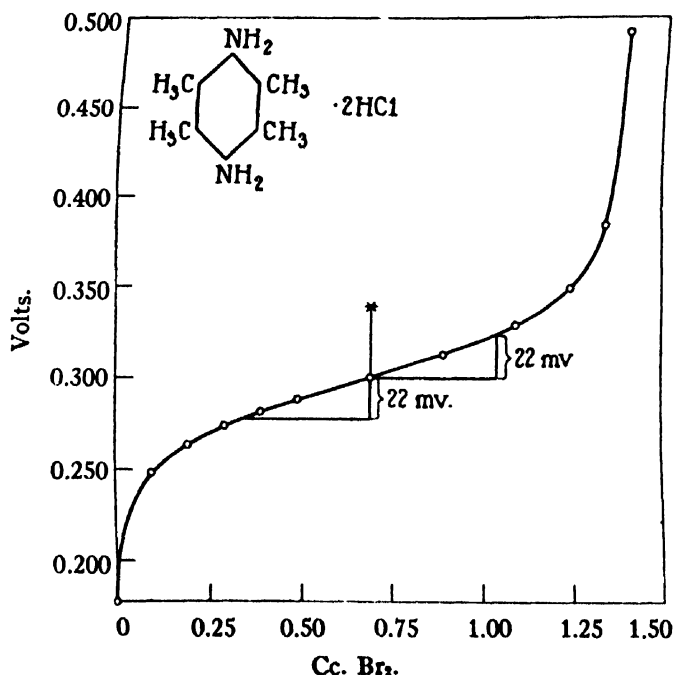
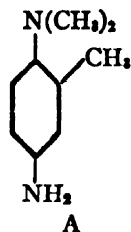


FIG. 1.—Durene diamine bihydrochloride, 50 cc. of 0.001 *M* in a solvent consisting of 8 volumes of methanol + 2 volumes of aqueous acetate buffer of *pH* 4.62 (apparent *pH* of the mixture is 6.21) is titrated with aqueous bromine. Potentials are constant throughout the titration. The titration curve indicates a two-step oxidation with the formation of an intermediate radical (which is yellow). The curve is symmetric, the index potential 22 mv. Hence, the semiquinone formation constant $k = 1.0$; maximum ratio of semiquinone to total substance is 0.33. In this case both the radical and the diimine are stable, at any rate within the period necessary for titration. The initial solution is colorless; it turns yellow during the titration and fades out again at the end-point of titration.

reaction takes place greatly depends on the initial concentration of the diamine. When this is varied, keeping constant the amount of bromine used for the oxidation, the rate of the irreversible reaction is greatly diminished as the concentration of the diamine is increased. According to reaction (1) the amount of diimine formed is decreased as the concentration of the diamine is increased. This experiment

shows that the irreversible reaction is caused essentially by the irreversible changes of the diimine present and that the free radical is much more stable in this solvent than is the diimine. The diimines which have been prepared by Willstätter³ are all extremely unstable in organic solvents not perfectly water free. When methanol—even containing as much as 20% water—is chosen as a solvent, the lifetime of the radical is much longer, but also here the lifetime of the radical is increased as the initial concentration of the diamine is increased. The concentrations of the diamine and of bromine being properly chosen, the lifetime of the radical may be extended to more than a whole day. By extrapolation we may infer that the direct lifetime of this radical is very long, and its apparent lability for the greatest part is due to the fact that the equilibrium constant of the reaction (1) is such as to cause the two steps of oxidation greatly to overlap. This is a case where the direct lifetime proper of the radical can be proven to be quite long, although at first glance the radical may have the appearance of being very labile.

Third Case.—When the same experiment is performed with the diamine (formula A) the radical has a half lifetime of about ten minutes in an aqueous acetate buffer. In this case the lifetime is not influenced at all by varying the initial concentration of the diamine. This radical itself has a restricted lifetime and its breakdown is not caused indirectly by the diimine which may be in equilibrium with it. The direct disappearance of the radical is so fast that the rate of the indirect breakdown, if there be any, plays no rôle.



Fourth Case.—When the same experiment is performed with symmetrical dimethyldiaminodurene, no radical is formed under any

(3) R. Willstätter and E. Mayer, *Ber.*, **37**, 1494 (1904); R. Willstätter and A. Pfannenstiehl, *ibid.*, **37**, 4605 (1904); R. Willstätter and H. Kubli, *ibid.*, **42**, 4135 (1909).

conditions. In this case the corresponding diiminium compound, although not perfectly stable for any length of time, is yet stable enough to allow a satisfactory potentiometric titration in 80% methanol. The shape of the titration curve confirms that there is no intermediate radical formed at all. Here it is meaningless to speak

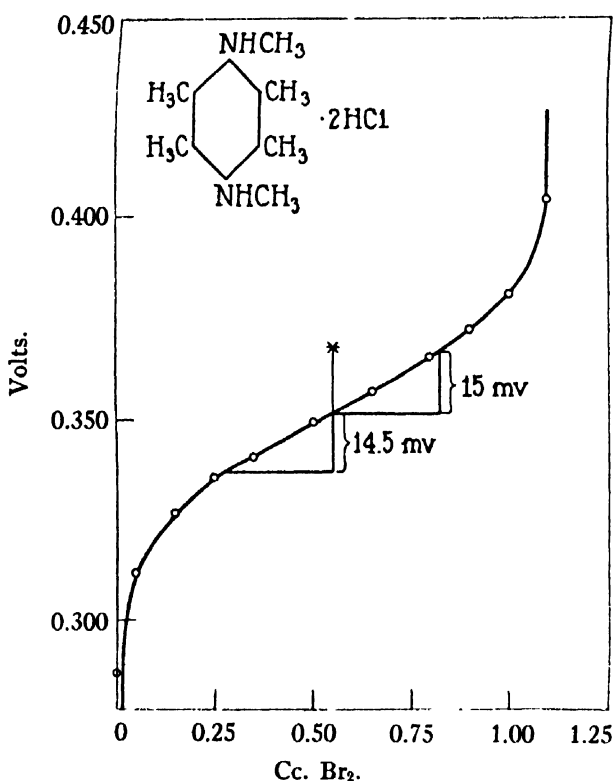


FIG. 2.—Symmetrical dimethyldurene diamine dihydrochloride. Conditions of titration the same as in Fig. 1. Here also a full titration curve is obtained. The index potential is between 14 and 15 mv., indicating that no intermediate radical is formed, in agreement with the fact that no trace of any intermediate colored compound is visible during the titration. The point * in this and all the other curves marks the end of the first step, where 1 atom of bromine per molecule of the diamine has been added.

of the lifetime of the radical. The fact that it is never formed to any measurable extent is itself an expression of its utmost instability, although it must be conceded that here the term "instability" has not precisely the same meaning as in the other cases.

These four examples cover all cases which may be actually en-

countered. Using critically such tests for stability, the results obtained may be summarized as follows:

The radical derived from *p*-phenylenediamine may be considered as the parent substance. Considering its stability proper according

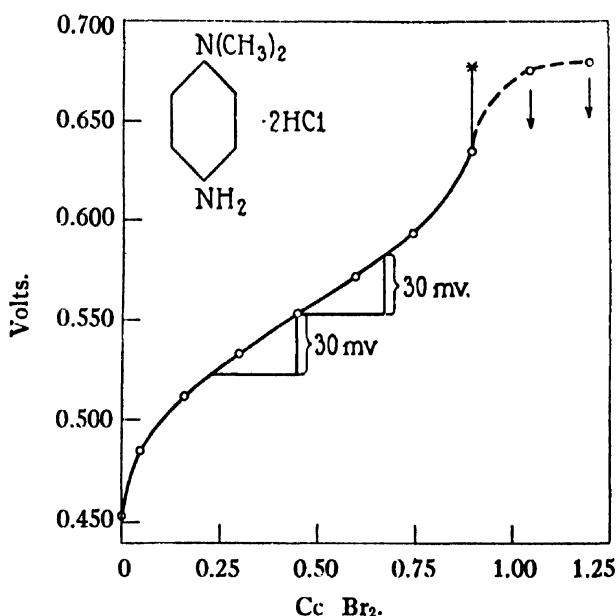
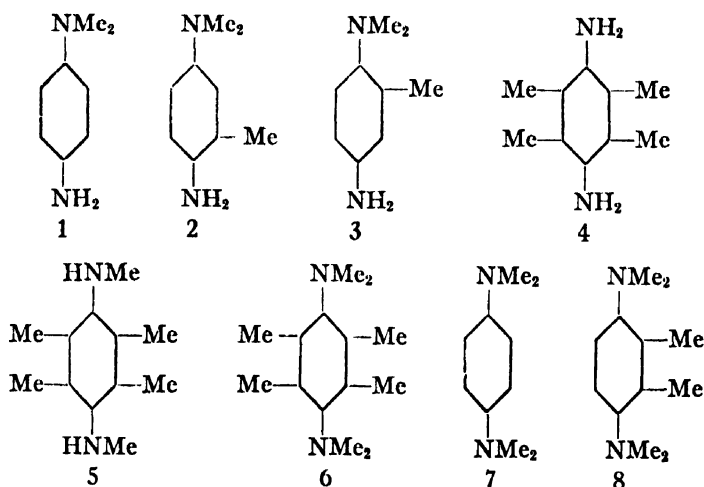


FIG. 3 — Unsymmetrical dimethyl-*p*-phenylenediamine bihydrochloride, 50 cc. of 0.001 *M* solution in a mixture of 8 volumes of methanol and 2 volumes of 0.05 *M* aqueous acetic acid (apparent *pH* of mixture is 3.1) are titrated with aqueous bromine. Potentials are nearly steady only to the end of the first step which is marked with a star (*). During the second step, potentials show considerable drift, as indicated by arrows. Since there is a slight jump at the end of the first step this first step can be treated approximately as a univalent step with no overlapping. The index potential of this univalent step should be 28.6 mv. It is found to be 30 mv., which is in sufficiently good agreement considering the limits of accuracy under the conditions prevailing. Hence, the curve suggests two successive univalent steps of oxidation with no, or very little, overlapping; the first step is reversible; the second step is irreversible due to the spontaneous breakdown of the diimonium compound.

to the above criterion, it is a rather stable compound. Its well-known instability in aqueous solution is essentially an indirect one. Its direct stability can be modified by substitutions. These may be either at the amino groups or at the ring. Considering first compounds with the amino groups unsubstituted, it can be generally

stated that substitution of the H atoms in the ring by methyl groups has no influence on the stability exceeding the limits of error of the method. Comparing the compounds not methylated at the ring but methylated at the amino groups, methyl groups appear to increase the stability. The tetramethylamino compound is the most stable of the series. There is, however, no cogent proof that these differences may not be due to differences of the indirect labilities. When both the amino groups and the H atoms of the ring are substituted, the effect largely depends on the particular structure. A methyl group at the ring in ortho position to an unmethylated amino group has sometimes very little effect, sometimes more, and, if any at all, a destabilizing effect. However, if a methyl group is in ortho position to a methylated amino group, it always has a distinct destabilizing effect; two methyl groups, especially if both are ortho to a methylated amino group, decrease the stability to an enormous extent. It may be added that the lifetime of any of these compounds is not affected by diffuse daylight.

To give some examples, the radical derived from 1 (Wurster's red) is a very stable one; 2 is almost as stable; but 3 has a half-lifetime only of less than five minutes in an aqueous solution; the radical derived from 4 is very stable; 5 and 6 do not establish any radicals at all; 7 gives a very stable radical, perhaps the most stable of all; whereas the radical derived from 8 fades out in an aqueous solution within a few minutes.



Substitutions of one single H atom at the ring other than by methyl such as by OCH_3 , Cl , SO_3H , all have a distinct destabilizing effect.

The decay of the radical can be observed most easily from the change of its color. In some cases the color simply fades out, in others a new dyestuff may be formed due to condensations. In

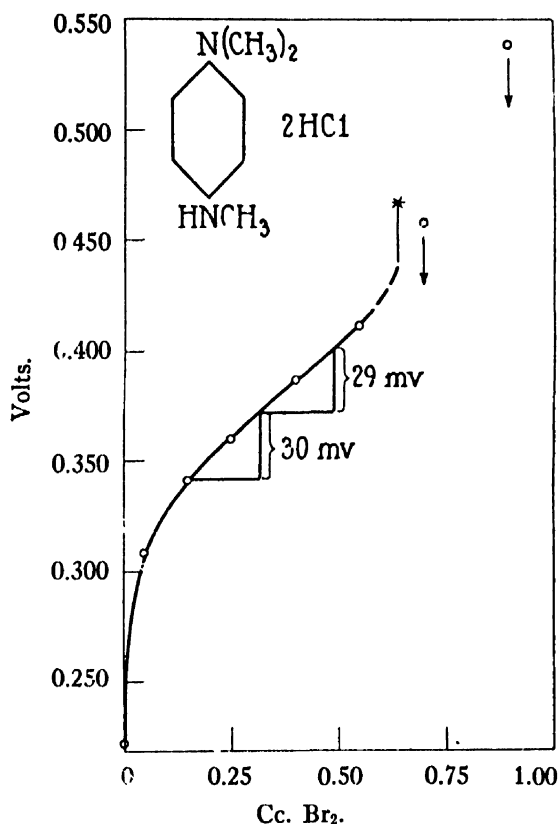


FIG. 4.—Trimethyl-*p*-phenylenediamine dihydrochloride Conditions of titration similar to those of Fig. 3. Potentials are fairly stable during the first step of oxidation; the index potential corresponds practically to a univalent oxidation (theoretically, 28.6 mv.) and no overlapping with the second step. During the second step of oxidation potentials drift rapidly, showing the instability of the diimine.

addition, potentiometric measurements give some indications as to the stability of the radical. If both the radical and the diimine are stable, a potentiometric titration of the diamine with bromine gives a full reproducible potentiometric titration curve up to the end-point of titration. Diaminodurene is the only example of this kind

among the compounds investigated.⁴ If the radical is stable and the diimine is unstable, steady potentials are obtained only in the first stages of the titration as long as there is no appreciable overlapping of the two steps of oxidation. If the radical itself is unstable, the potentials will not be steady even in the first stages of the titration. Many examples of these types of titration curves were obtained, and some are reproduced graphically in the experimental part. Thus a critical discussion of the potentiometric titration curves gives an

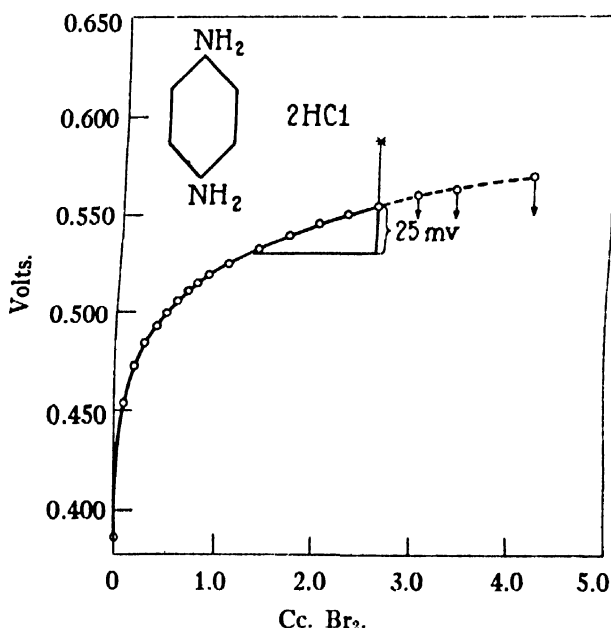


FIG. 5.—*p*-Phenylenediamine dihydrochloride. Conditions of titration similar to those of Fig. 3. The potentials are almost, although not perfectly, steady in the first part of the titration and then begin to drift rapidly.

indication as to the comparative stability of various radicals. The results of the two methods never showed any discrepancy. The limiting case of instability of a radical is the case where no radical is formed at all. If in such a case the diimine happens to be a relatively stable compound, the titration curve should have the shape of a reversible bivalent titration without any intermediate step. Such a

(4) Another example of this kind is symmetrical diphenyl-*p*-phenylenediamine [Michaelis and Hill, *THIS JOURNAL*, **55**, 1481 (1933)]. Aromatic substituents are not included in the present paper.

case is realized for symmetric dimethyldiaminodurene. If a radical is not formed, and the corresponding diimine compound is not stable either, no steady potentials can be obtained at all during the titration. Such a case is realized in tetramethyldiaminodurene.

2. *Absorption Spectra.*—The absorption bands for the radicals of the diimines substituted in the amino group, but not in the ring, have been described by Piccard. There are two distinct bands and, if the

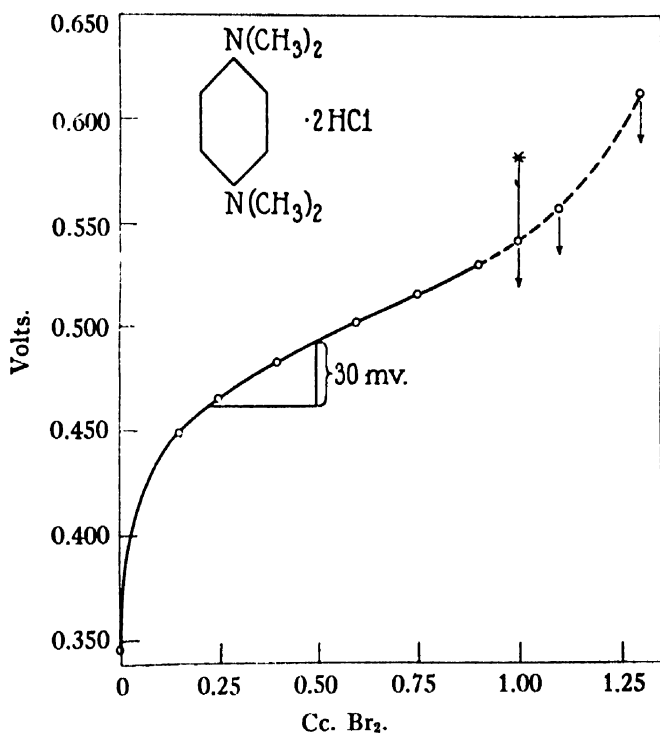


FIG. 6.—Symmetrical tetramethyl-*p*-phenylenediamine dihydrochloride under the same conditions as in Fig. 3. This curve is similar to that of Fig. 3 except that the potentials begin to drift a little earlier.

amino groups are in part or all methylated, a third less conspicuous one toward the shorter wave lengths. Each methyl group substituted in the amino group has a bathochromic effect, displacing the bands toward longer wave lengths. The molar absorption is distinctly greater for the tri- and tetramethyl compounds than for the others. There is scarcely any difference in the effect of methyl and ethyl; however, carboxymethyl (CH_2COOH) has a stronger bathochromic effect.

Comparing compounds not methylated at the amino groups, the effect of gradually attaching methyl groups at the ring is no bathochromic effect at all, but consists in changing the pattern of the bands as follows (Fig. 8). Without any methyl in the ring the first peak (the one toward the shorter wave lengths) is higher than the second; with one methyl, the two peaks are blurred into one; with two and more methyls, the first peak is decreased in height, the second increased; this effect is greatest in the durene compound.

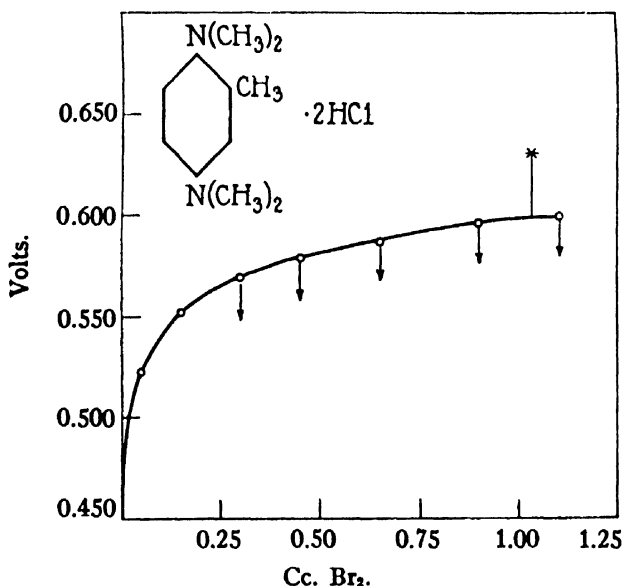


FIG. 7.—Symmetrical tetramethyldiaminotoluene dihydrochloride. Same conditions as in Fig. 3. This curve is one example for a much more unstable radical than Figs. 5 or 6. Potentials drift even in the first stages of titration.

Comparing the effect of substitutions at the ring in compounds with one or more methyls at the amino groups, the effect of one methyl in the ring consists usually in blurring the pattern; the two bands overlap to a greater extent (Figs. 10, 11, 13). Two methyl groups in the ring make the absorption pattern rather diffuse, (Figs. 9, 13) showing a weak, broad, blurred band. As the bands are blurred the total molar absorption is diminished.

3. *Interpretation.*—The fact that such radicals are capable of existence at all can be attributed to a particular symmetry of structure resulting in resonance. The molecule has an odd number of electrons; the electrons may be paired in various ways, leaving always one un-

paired electron. The odd electron has no fixed place, but there is resonance among various limiting states. Each of them may be considered as contributing a share to the resonating or mesomeric state but none of them can exist as a separate, tautomeric structure for any conceivable length of time. Those two limiting states which

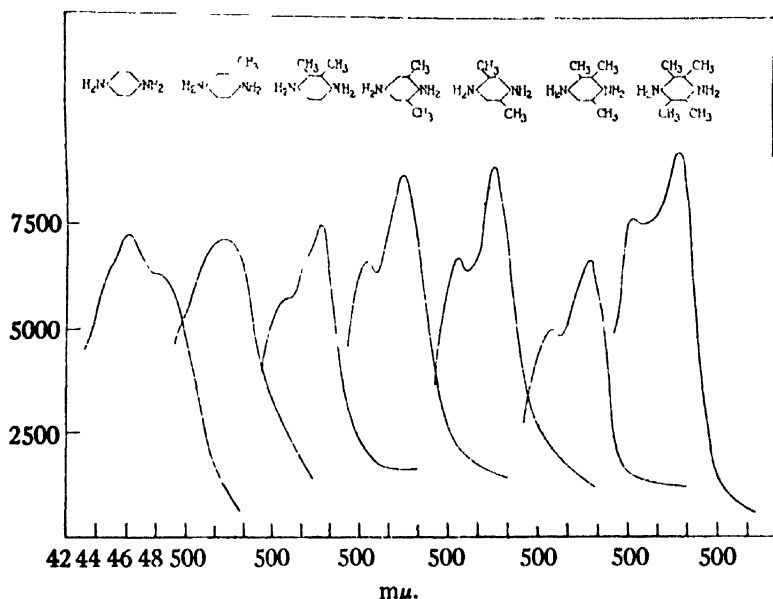
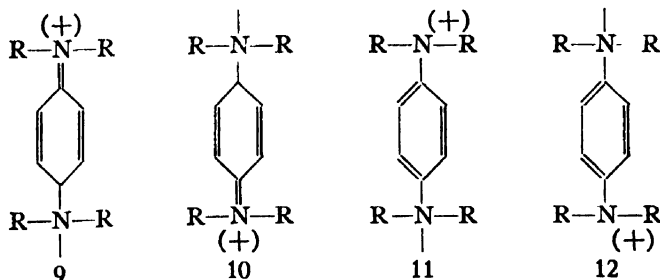


FIG. 8.—Molar absorption plotted against wave lengths for radicals of diamines methylated at the ring, but not at the amino groups. In reality all the curves overlap. All curves should be imagined as displaced horizontally to the left so that the points marked "500 mμ" coincide.

may be considered as the main contributors to the resonating state may be symbolized⁵ by 9 and 10. There are more configurations of which 11 and 12 are examples. Resonance among these various



(5) In these formulas letters are atomic kernels, dashes are pairs of electrons, which represent a chemical bond only if they connect two kernels, and the dot is a single electron.

structures can take place only if the six carbon atoms of the ring, the two nitrogen atoms and the four atoms attached to the two nitrogen atoms, all are located in one plane. Structures such as 9 or 10 resemble a quinone structure with an odd electron at a carbon atom, while structure 11 or 12 represents a benzenoid structure with an odd electron at a nitrogen atom. Radicals of this type may be designated

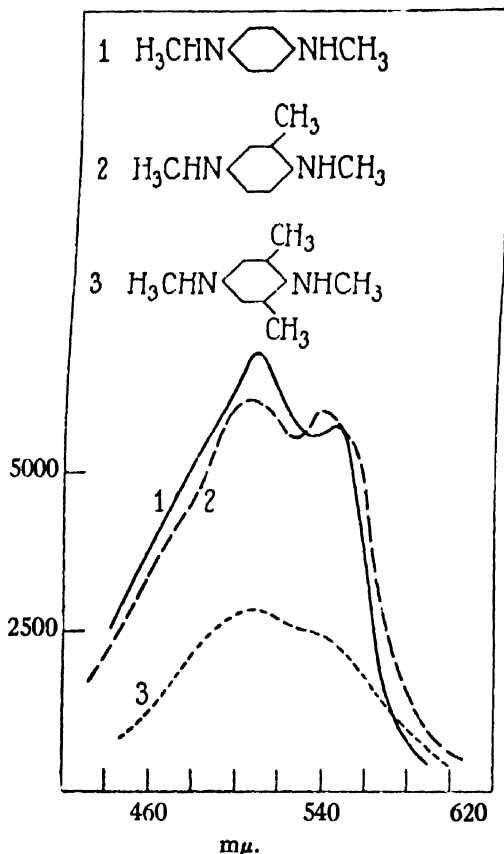


FIG. 9.—Molar absorption plotted against wave lengths for radicals of three symmetrical dimethyl diamines differing by substitution at the ring.

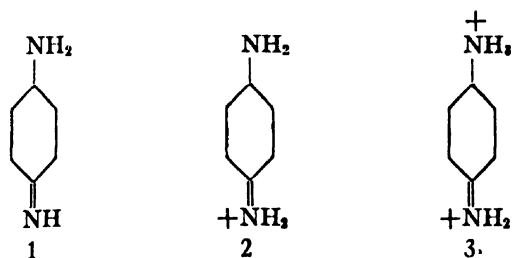
as semiquinones. The ambiguity as to the benzenoid and the quinonoid structure, or the meriquinoid character which is responsible for the color, lies within a single ring,⁶ in contrast to ordinary organic dyestuffs, such as indophenols or triphenylmethane dyes, which are not radicals, and in which this ambiguity is distributed over two (or three) rings. It is the aim of this paper to show that all those

(6) G. Schwarzenbach and L. Michaelis, *THIS JOURNAL*, **60**, 1667 (1938).

radicals showing little stability possess a structure which counteracts a coplanar arrangement such as would be necessary for resonance.

Although not all, yet some of the effects of substitution may be accessible to a rational interpretation at the present time. It is very remarkable that methyl groups in the ring have such a strong destabilizing effect on the radical if the amino groups also are methylated. This effect can be accounted for as follows. In the resonating state, the bond between N and C is something intermediate between a single and a double bond. The properties of such a quasi double bond will approach those of a double bond and restrict the free rotability of the amino group around this bond as the axis. The two atoms linked by the double bond and the four atoms attached to them must lie in one plane. This plane must be also the plane of the benzene ring. If such a coplanar arrangement requisite for resonance is hampered, stability is diminished.⁷

Some Additional Remarks on the Solvent and on pH.—The stability of all of these radicals depends on pH. Even the most stable radicals are stable only within a certain pH range, which in aqueous solution is usually from pH 3.5 to 6. Outside this range all of these radicals become unstable. This is easily accounted for by taking into consideration that among the three possible states of ionization of the radical only formula 2 can exhibit the resonance necessary for the stability. This state, or rather the resonance system of which formula 2 is one of the limiting states, secures whatever stability may be inherent in such an unsaturated compound. A pH very suitable for



stability in most compounds in aqueous solution is 4.6; in 80% methanol it is about pH 3 for most of the compounds, and about pH

(7) Compare: L. Pauling and G. W. Wheland, *J. Chem. Phys.*, **1**, 362 (1933); E. Hückel, *Z. Physik*, **83**, 632 (1933); *Z. Elektrochem.*, **43**, 752 and 827 (1937).

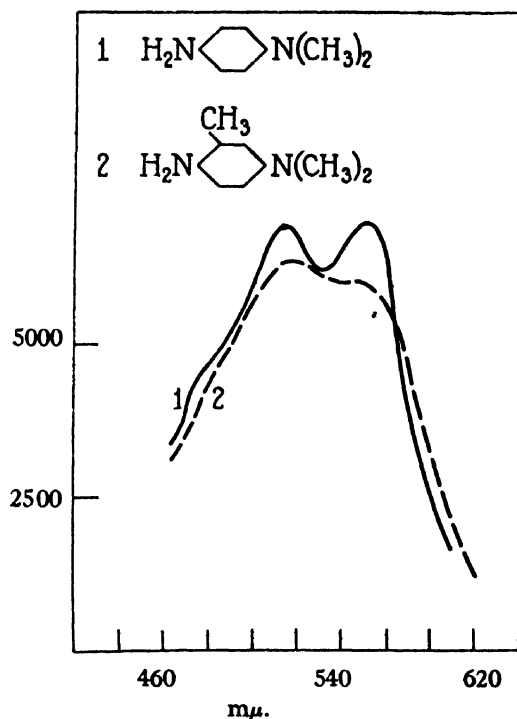


FIG. 10.—Another example of the influence of substitution at the ring upon the pattern of the absorption spectrum.

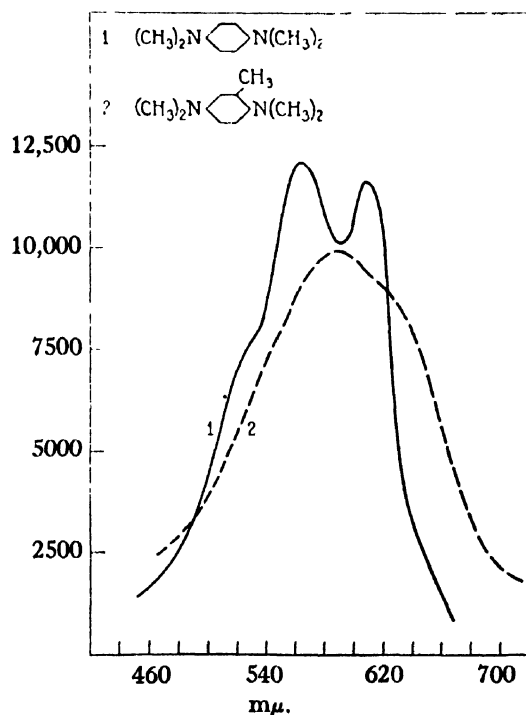


FIG. 11.—A further example of the influence of substitution at the ring upon the pattern of the absorption spectrum.

6 for the durene compounds. One easily understandable exception is the radical derived from tetracarboxymethyl-*p*-phenylenediamine, which has its best stability in aqueous solution at *pH* 7. At a lower *pH*, not all of the four carboxyl groups are ionized. If there be any

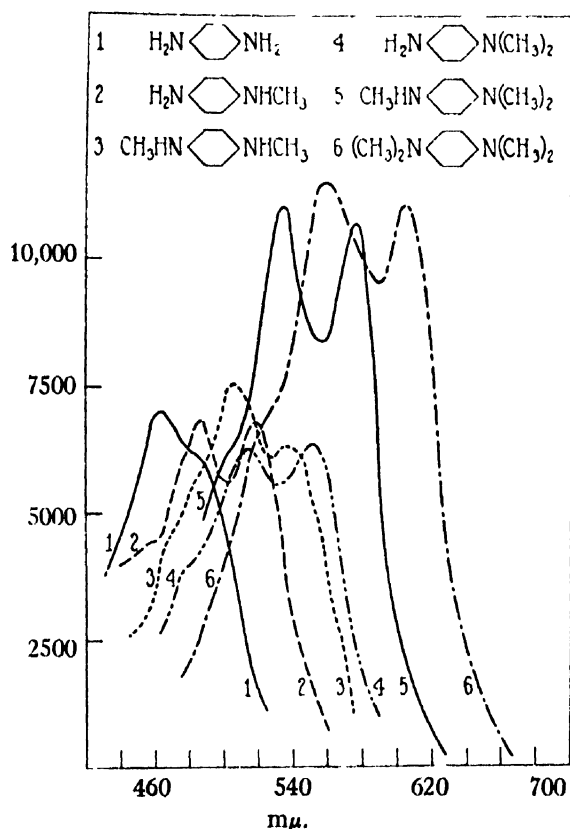


FIG. 12.—Molar absorption plotted against wave lengths for radicals derived from diamines substituted at the amino group but not at the ring. The tetracarboxymethyl derivative is not included in this graph. It is shown in Fig. 13, the last curve, dotted line.

asymmetry with respect to the free electrical charges at these carboxyl groups, resonance will be counteracted.

It has been pointed out before that the radical of *p*-phenylenediamine is very much more stable in methanol than in water. As the amino groups are progressively methylated, the difference of the stability in aqueous and in alcoholic solutions becomes much smaller, and in the tetramethyldiamino compound the radical is perfectly stable both in water and in alcohol. This is quite different from the

behavior of the diimines which are all quite unstable in water, and even in alcohol if not quite water free; whereas the stability of the radicals in methanol is scarcely diminished even if the alcohol contains 20% of water. The only diimines which in such a solvent have

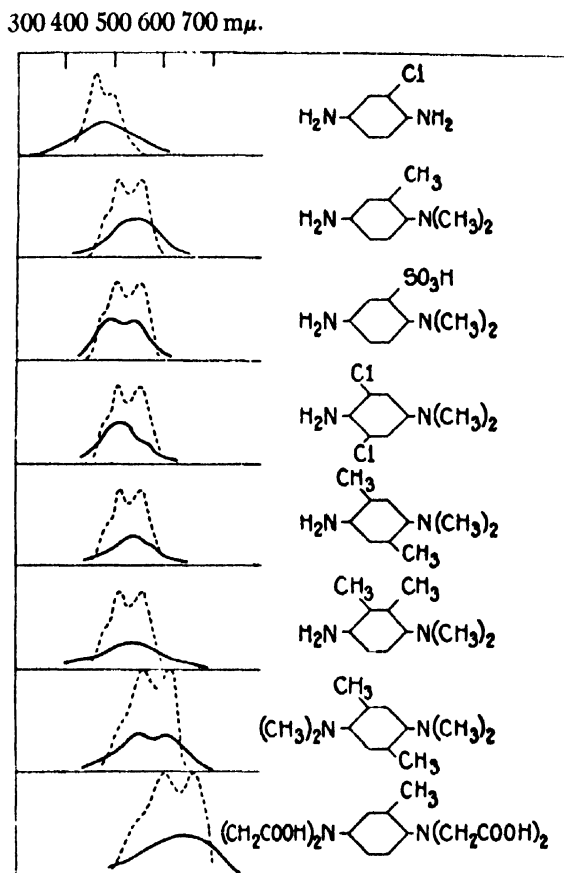


FIG. 13.—Rough sketches of absorption spectra of some compounds substituted at the benzene ring which are not stable enough for accurate measurements of the absorption (drawn out curves). They are obtained by comparing in the hand spectroscopie the freshly prepared solutions with those obtained by the homologous, more stable compound with no substitution at the ring. The latter is always plotted in the dashed curve, reproduced from the accurate measurements with the spectrophotometer.

a sufficient stability to allow reproducible potentiometric titration curves throughout both steps of oxidation are the diimine compounds derived from diaminodurene and symmetrical dimethyldiaminodurene.

The authors are greatly indebted to Professor Linus Pauling for comments and advice with respect to some of the theoretical discussions.⁸

EXPERIMENTAL

1. Preparation of the Compounds.—All of the *p*-diamines used in this work were prepared as the dihydrochlorides. 1,4-Diaminobenzene, 1,4-diamino-2-methylbenzene and 1-amino-4-dimethylaminobenzene were obtained as commercial preparations and purified by dissolving in boiling methanol or boiling methanol containing 10% of water. The solution is decolorized with Darco G. 60 and the hydrochlorides reprecipitated by addition of 5 to 10 volumes of acetone and about 5% volume of concentrated hydrochloric acid.

The symmetrical *N,N'*-dimethyl derivatives, 1,4-di-(methylamino)-benzene, 1,4-di-(methylamino)-2-methylbenzene, 1,4-di-(methylamino)-2,6-dimethylbenzene and 1,4-di-(methylamino)-2,3,5,6-tetramethylbenzene were all prepared similarly. For example, 12 g. of 1,4-diaminobenzene dihydrochloride in 50 cc. of dry pyridine is heated with 26 g. of toluenesulfonyl chloride at 100° for one and one-half hours. The product is precipitated by pouring the mixture into water and is recrystallized by dissolving in dilute sodium hydroxide solution and adding acetic acid. This ditosyl derivative is methylated in aqueous alkali by the use of excess dimethyl sulfate and the crystalline product is filtered off and washed with dilute alkali. The tosyl groups are removed by warming this product at 90° in a mixture of 30 cc. of acetic acid and 60 cc. of concentrated sulfuric acid until a clear solution results and then for about two hours more. The mixture is diluted with water, made alkaline and extracted with ether. The base is dissolved in 300 cc. of acetone and the dihydrochloride precipitated by the addition of 6 cc. of concentrated hydrochloric acid. It is recrystallized by dissolving in hot methanol and adding 5% volume of concentrated hydrochloric acid and 5 volumes of acetone.

1-Amino-4-methylaminobenzene was prepared from methylacetanilide (55 g.) by nitration in a cold solution of 55 cc. of acetic acid and 110 cc. of sulfuric acid with 19 cc. of fuming nitric acid (d. 1.5); reduction of this nitro compound with stannous chloride in concentrated hydrochloric acid and hydrolysis of the resulting product by heating in this acid mixture under reflux for five hours. The base is separated and the dihydrochloride precipitated from acetone as above.

(8) Professor L. Pauling has drawn our attention to the following analogous case of steric hindrance on resonance: Birtles and Hampson [*J. Chem. Soc.*, 10 (1937)] show that the magnitude of the electric dipole moment of nitrobenzene can be accounted for by resonance due to coplanar arrangement of the NO₂ group and the benzene ring. In nitrodurene this effect of the NO₂ group is strongly diminished.

1-Methylamino-4-dimethylaminobenzene was prepared from 1-amino-4-dimethylaminobenzene by tosylating, methylating and hydrolyzing as for the N,N'-dimethyl derivatives.

A number of the desired compounds were prepared by coupling the appropriate amino compound with diazotized sulfanilic acid, recrystallizing the resulting azo compound and reducing it in boiling water containing a trace of acid by means of zinc dust.⁹ By this method there were prepared 1-amino-4-dimethylamino-3-methylbenzene, 1-amino-4-dimethylamino-2-methylbenzene, 1,4-diamino-2,5-dimethylbenzene, 1-amino-4-dimethylamino-2,5-dimethylbenzene, 1,4-diamino-2,6-dimethylbenzene, 1,4-diamino-2,3-dimethylbenzene and 1-amino-4-dimethylamino-2,3-dimethylbenzene.

Pseudocumene was prepared in pure form from a technical product by the method of Jacobsen.¹⁰ The pseudocumene sulfonic acid was recrystallized twice by dissolving in water and adding an equal volume of concentrated hydrochloric acid. The pseudocumene, b. p. 166–167°, obtained from this sulfonic acid was nitrated by the method of Schultz¹¹ and reduced to pseudocumidine. The subsequent transformation to 1,4-diamino-2,3,5-trimethylbenzene followed the method of Smith.¹²

Most of the N,N'-tetramethyl compounds were prepared either from 1,4-diamino or 1-amino-4-dimethylamino dihydrochlorides by heating with approximately a 50 to 100% excess of methanol in a sealed tube at 150° for eight hours. The free bases were separated in ether or chloroform and distilled at about 1 mm. pressure. In most cases no attempt was made to crystallize the resulting oily bases but these were converted directly in acetone to their dihydrochlorides.

Methylation of diaminodurene with methanol at 150° led, however, to the symmetrical N,N'-dimethyl derivative (m. p. of the base 93°) identical with that obtained by the method described above for preparing N,N'-dimethyl derivatives. This N,N'-dimethyldiaminodurene with nitrous acid in aqueous acid solution gives a dinitroso derivative quite smoothly (m. p. 212°). However, by refluxing diaminodurene in a methanol-water mixture with methyl iodide and sodium carbonate, tetramethyldiaminodurene can be obtained directly (m. p. of the base 67°).

1,4-Tetraethyldiaminobenzene and 1-dimethylamino-4-diethylaminobenzene were prepared from 1,4-diaminobenzene and 1-dimethylamino-4-aminobenzene, respectively, by heating 8 g. of the hydrochlorides in sealed tubes with 15 cc. of absolute ethanol, 1 g. of calcium chloride, 1 g. of cuprous chloride and 1 g. of sodium bromide. The bases are distilled and the hydrochlorides recrystallized from acetone as usual.

(9) E. Noelting and G. Thesmar, *Ber.*, **35**, 628 (1902).

(10) O. Jacobsen, *Ann.*, **184**, 199 (1877).

(11) G. Schultz, *Ber.*, **42**, 3606 (1909).

(12) L. I. Smith, *THIS JOURNAL*, **56**, 472 (1934).

2,5-Diaminobenzenesulfonic acid monohydrochloride was prepared by reduction of 2-amino-5-nitrobenzenesulfonic acid with stannous chloride and recrystallization of the product by dissolving in boiling water and precipitating with an equal volume of concentrated hydrochloric acid.

2-Dimethylamino-5-aminobenzenesulfonic acid monohydrochloride was prepared by heating 1-chloro-5-nitrobenzenesulfonic acid with dimethylamine at 100° for two hours and reducing the resulting product with stannous chloride. The tin is removed with hydrogen sulfide and the hydrochloride is recovered by evaporation. It is recrystallized by dissolving in a methanol-water mixture and precipitated with acetone.

The tetracarboxymethyl compounds were prepared as described previously.¹⁸

2. Compounds not Methylated at the Amino Groups.—The properties of the radicals derived from *p*-phenylenediamine and its homologs substituted at the ring but not substituted at the amino groups are summarized in Table I.

These compounds are more difficult to judge than the others, because they show a greater overlapping of the two steps of oxidation. If the diimine is relatively stable (as in no. 7), the radical is perfectly stable also. If the diimine is very unstable (as in no. 1), it is always obvious that the radical is at least very much more stable than the diimine. The apparent instability of the radical is either in part or entirely an indirect one as can be proved for all these compounds with the "concentration test" described above. Using a higher concentration of the diamine, but the same amount of bromine, the lifetime is considerably longer than given in the table.

As the yellow color of these radicals disappears in time it either may be replaced by a different color due to condensation (*e. g.* in no. 1), or simply fade out if such condensations are inhibited owing to substitutions at the benzene ring (as in no. 3 or 7). If a condensation to a colored substance (pink or violet, according to the particular compound) takes place, the lifetime of the radical may be greatly underestimated since its yellow color is superseded by the deeper color of the secondary reaction product. The duration of the yellow color is greatest in pure methanol. However, in order to make possible a variation in *pH* so as to find out its optimum condition, and also in order to make possible the maintenance of a given *pH* during the corresponding potentiometric titration experiment as shown in the last column, the experiments as tabulated were performed in a solvent consisting of 80% methanol and 20% of an aqueous buffer. The latter was chosen so as to give the greatest stability. It was 0.05 *M* aqueous solution of acetic acid, producing in the mixture an apparent *pH* of about 3, for the compounds nos. 1 to 5; it was acetate buffer (*pH* 4.6) producing an apparent *pH* about 6 for compounds nos. 6 and 7.

In order to demonstrate how much the two steps of oxidation overlap in these compounds, the potentiometric titration curve for no. 7 (Fig. 1) may be chosen,

(13) L. Michaelis and M. Schubert, *J. Biol. Chem.*, **106**, 331 (1934).

TABLE I

The first column gives the diamine from which the radical is derived by partial oxidation with bromine. All compounds were prepared as bihydrochlorides, except for the two last ones. All compounds mentioned in the table gave satisfactory analyses for N and Cl.

		Color	Lifetime	m μ	Type	Potential behavior in Br $_2$ titration in solvent indicated ^a
<i>p</i> -Phenylenediamines						
1	Unsubstituted diamine (1, 4)	Yellow (turning blue)	4-8 hrs.	462	Sharp	Steady to 75% of 1st step
2	2-Methyl-	Yellow (turning brown)	4-8 hrs.	466	Broad	Steady to 75% of 1st step
3	2, 5-Dimethyl-	Yellow (fading out)	2-3 days	452	Sharp	Steady to 75% of 1st step
4	2, 6-Dimethyl-	Yellow	12 hrs.	452	Sharp	
5	2, 3-Dimethyl-	Yellow	12 hrs.	452	Sharp	
6	2, 3, 6-Trimethyl- ^a	Yellow	2-3 days	452	Sharp	Steady to 70% second step
7	2, 3, 5, 6-Tetramethyl- ^a	Yellow	2-3 days!	452	Sharp	Steady throughout both steps
8	2-Methoxy-	Yellow	15 min.	475	Broad	Unsteady
9	2-Sulfonic acid- ^b	Yellow-pink	2-3 sec.	Unsteady
10	2-Chloro-	Pink-yellow	2-3 min.	475	Broad	Unsteady
11	2, 6-Dichloro-	Yellow	2-3 sec.	...	Diffuse	Unsteady
Monomethyl- <i>p</i> -phenylenediamine						
12	Unsubstituted	Orange-pink	1 day	483	Sharp	Steady to 100% of 1st step
Symmetrical Dimethyl- <i>p</i> -phenylenediamines						
13	Unsubstituted	Pink	1 day	508	Sharp	Steady to 100% of 1st step
14	2-Methyl-	Pink	1 day	505	Sharp	Steady to 100% of 1st step
15	2, 6-Dimethyl-	Pink	2 hrs.	505
16	2, 3, 5, 6-Tetramethyl- ^a	Colorless	No radical	Steady ^c

Unsymmetrical Dimethyl-*p*-phenylenediamines (1-Dimethylamino-4-aminobenzene)

		Red	> 7 days ^d	515	550	Sharp	Steady to 100% of 1st step ^a
17	Unsubstituted	Violet-red	5 min.	520		Broad	Unsteady
18	2-Methyl-	Red	2 days!	515	550	Steady to 100% of 1st step
19	3-Methyl-	Violet-pink	1 min.	490	600	Broad	Unsteady
20	2,5-Dimethyl-	Violet-pink	20 sec.	470	510	Broad	Unsteady
21	2,3-Dimethyl-	Pink	2 min.	495	530	Unsteady
22	2-Sulfonic acid						

Trimethyl-*p*-phenylenediamine

	Purple-red	> 7 days	536	578	Sharp	Steady to 90% of 1st step
23	Unsubstituted					

Symmetrical Tetramethyl-*p*-phenylenediamines

	Blue	Weeks!	560	606	Sharp	Steady to 100% of 1st step
24	Unsubstituted	1 day	590		Broad	Steady to 30% of 1st step
25	2-Methyl-	2-3 hrs.	540	560	Diffuse	Unsteady
26	2,5-Dimethyl-	1 min.	Diffuse		Broad	Unsteady
27	2,3-Dimethyl-		Unsteady
28	No radical formed		Unsteady
29	2,3,6-Trimethyl-					
29	2,3,5,6-Tetramethyl-					

Miscellaneous *p*-Phenylenediamines

	Blue	14 days	560	606	Sharp	Steady to 100% of 1st step
30	<i>unsym.</i> -Dimethyl-diethyl- ^g	14 days	560	606	Sharp	Steady to 100% of 1st step
31	Tetraethyl-	14 days	560	606	Sharp	Steady to 80% of 1st step
32	Tetracarboxymethyl- ^g	14 days	596	645	Sharp	Unsteady
33	Salt of 2-methyl-tetra-(carboxymethyl)-	5 min.	Very diffuse spectrum			

^a Dissolved in 8 volumes of methanol + 2 volumes aqueous acetate buffer, pH 4.6. ^b Dissolved in aqueous acetate buffer, pH 4.6. In all other cases, where no letter is indicated, the solvent is 8 volumes of methanol + 2 volumes of 0.005 *N* aqueous solution of acetic acid. ^c Curve for a simple bivalent oxidation without intermediate step. ^d In absence of O₂, color and intensity change relatively slowly. After five days, the intensity had diminished appreciably, while the intensity of Wurster's blue and the trimethyldiamine had not changed under the same conditions. In aqueous acetate buffer (pH 4.6) even in the absence of oxygen, the solution turns to a dirty violet color in one day, the original bands being just visible. ^e Potential jumps to second step, and becomes unstable. ^f Indistinguishable from the tetramethyl compound. ^g Aqueous phosphate buffer at pH 7.26. ^h The potentials are, of course, never steady in the strictest sense of the word. There is also a certain arbitrariness in the stage of oxidation where a potential begins to show a distinct drift. The indications in this column can, however, be used for comparative purposes.

which shows steady potentials throughout both steps of oxidation. From this curve it can be derived¹⁴ that the semiquinone formation constant is equal to 1.0. Hence, in the mid-point of titration, where the concentration of the radical is maximum, not more than 33% of the substance exists as radical. When only 0.1 atom of bromine is added to 1 molecule of the diamine, 8% of the substance is present as radical, and still almost 1% as diimine: there is always appreciable overlapping. In aqueous solution the overlapping seems to be even greater, although definite figures cannot be given on account of the instability of the potentials.

So it is evident that the direct stability of the radicals nos. 1 and 7 is much greater than the lifetime in column III seems to indicate. Whether the differences of lifetime within this group are real differences in direct stability or indirectly caused by differences in the stabilities of the diimines, is difficult to decide. There is no doubt that the diimines, although all of them are unstable, differ greatly in stability among themselves according to the number and positions of the methyl groups at the ring which influence the ease and possibility of secondary reactions. For instance, the diimine no. 7 should be, and really is, much more stable than no. 1. It is not certain whether this difference is reflected also in the corresponding radicals.

In contrast, in the compounds 8 to 11, the lifetime of the radical is so short under any condition that the radical itself must be considered as unstable.

3. *Compounds Methylated at the Amino Groups* (Table I, from no. 12 to the end). (a) *Benzene Derivatives*.—The common feature of these compounds is the fact that the difference between the stability in aqueous and in alcoholic solution is much less conspicuous. This difference becomes smaller as the number of methyl groups at the nitrogen is increased. In the extreme case, for the tetramethylamino compound, the difference between water and alcohol as a solvent vanishes entirely. The fact that these radicals are much more stable in an aqueous solution than the unmethylated ones, may be attributed essentially to the fact that the overlapping of the two steps of oxidation is smaller. As a consequence the indirect lability of the whole system due to the hydrolysis of the diimine in aqueous solution is almost eliminated. Although full potentiometric titration curves cannot be obtained because of the lability of the diimines, such a curve as Figs. 3 and 4 indicates that in spite of the rapid drift of the potentials in the negative direction during the second half of titration, there appears a transient potential jump in the mid-point of titration. This is possible only when the semiquinone formation constant is essentially larger than 16, whereas in the unmethylated diaminodurene mentioned before this constant is 1.0.

The most suitable solvent to combine a sufficient stability of the potentials with a sufficient buffer capacity in order to maintain a constant pH during the

(14) Regarding the method of calculation see Michaelis and Schubert, *Chem. Rev.*, **22**, 437 (1938).

titration is 8 volumes of methanol and 2 volumes of 0.05 *M* aqueous acetic acid (apparent *pH* of the mixture, 3.1) but the stability is quite satisfactory also in an aqueous acetate buffer *pH* 4.6. Under proper conditions, all of these radicals are stable at least for several days. In the tri- and tetramethylamino compounds the radical does not fade out even over a period of many days; on the contrary, the color increases somewhat, in part due to further oxidation by exposure to the air, and to a slight extent even in the strict absence of oxygen, due to secondary dismutation of the traces of the diimine present. The nature of this peculiar decomposition of the diimine is of no interest for the present purpose.¹⁵ At any rate, all of these radicals are very stable compounds in great contrast to their corresponding diimines.

(b) *Compounds Substituted both at the Ring and at the Amino Groups.*—A single methyl group at the ring has no, or at least no conspicuous, effect on the stability of the radical, if it is ortho to an unmethylated amino group; however, it has a very decided destabilizing effect when it is ortho to a methylated amino group. This effect is a direct one and is not caused indirectly by the interference of the diimine, as can be shown by the test described before. In the ψ -cumene and durene compounds, no radical is established at all if the amino groups are methylated. This is true whether the corresponding diimine is stable or labile. In the symmetrical dimethyldiaminodurene, the diimine is rather stable in 80% methanol; in tetramethyldiiminodurene it is extremely labile, yet, in both cases, no radical is formed at all.

Of the many details presented in this table one item is worth special attention. No. 18 is considerably more labile than 17; correspondingly no. 26 is more labile than 25, but no. 26 is much more stable than 18. While in unsymmetrical dimethyldiaminobenzene the introduction of one CH₃ group in the ring ortho to the methylated amino group has a strong destabilizing effect, this effect is smaller for a corresponding substitution in tetramethyldiaminobenzene. In the latter case, the introduction of two methyl groups in the ring is necessary to exert a really

(15) For the understanding of this peculiar process it will suffice to describe the following observations. When a solution of dimethyl-*p*-phenylenediamine in acetate buffer is titrated very rapidly with just enough bromine to cause the red color of the radical to disappear again, and when the colorless solution is allowed to stand, the color of the radical reappears in a short time. The same is true for tri- and for tetramethyl-*p*-phenylenediamines. In the tetra compound there is a remarkable difference insofar as the radical which is secondarily reestablished is, according to its spectrum, that of the tri, instead of the tetra compound. This again is in conformity with Willstätter's observation that the tetramethyldiimonium compound, even in the solid state, easily loses one methyl group in the form of formaldehyde. The radical itself of the tetramethyl compound (Wurster's blue), has not the slightest tendency to lose a methyl group. This shows once more how much more stable is the radical than the diimonium compound.

strong destabilizing effect. Compare also the difference of this effect according to the position of the two methyl groups (nos. 27 and 28).

4. *Absorption Spectra*.—Ten cc. of a 0.01 *M* solution of the dihydrochloride in the same solvent as indicated in the tabulation, was mixed with 1 cc. of 0.001 *N* aqueous solution of bromine. The concentration of the radical was calculated according to the amount of bromine added, neglecting the overlapping of the two steps, which causes no appreciable error if the excess of unoxidized diamine is as large as in these experiments. A König-Marten's spectrophotometer was used for the readings.

SUMMARY

The radicals derived from aromatic *p*-diamines as modified by substitutions at the amino groups and at the benzene ring, by univalent oxidation, are compared with each other with regard to stability and color. The stability is measured approximately in terms of the lifetime in a properly chosen solvent. A distinction has to be made as to whether the disintegration is due to the direct breakdown of the radical ensuing from interaction with the solvent, or is caused indirectly by the lability of the diimine with which it always is in equilibrium. The indirect lability is most obvious in compounds not methylated at the amino groups. It may make the radicals appear to be much more labile than they really are. The direct spontaneous lability of the radicals is increased whenever there is a steric hindrance preventing the coplanar arrangement of the molecule necessary for resonance. This is the case when one or two methyl groups substituted in the benzene ring are in ortho position to a methylated amino group. Direct observations of changes in color are supported by potentiometric oxidative titrations of the diamines. The absorption spectra are recorded. It is especially noteworthy that diaminodurene forms a very stable radical, but that any methylation of its amino groups entirely prevents the formation of a radical, due to the steric hindrance preventing coplanar arrangement of the molecule.

CAUSES OF THE CESSATION OF GROWTH OF FIBROBLASTS CULTIVATED IN EMBRYO JUICE*

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PLATE 37

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Fibroblasts cultivated according to the flask technique in a plasma coagulum with embryo juice as nutrient fluid do not proliferate continuously. Generally, after 2 or 2½ weeks of cultivation the cells stop multiplying although only a small portion of the coagulum is covered with tissue. If a part of the tissue is transferred to a new coagulum, growth is resumed. If it is not so transferred, the cells degenerate. Investigators who have attempted to find the cause of this cessation of growth and subsequent degeneration of the cells, have generally attributed it either to changes that occur in the physical structures of the coagulum, or to the accumulation of toxic products therein.¹ Some of these investigators have cut out a section of the old coagulum in which fibroblasts had stopped growing and have filled in the aperture with fresh plasma. The renewed growth that resulted was taken as evidence that the cells were able to invade a newly formed coagulum but did not have the power to invade one that had aged.² Acting on this hypothesis, Mayer has developed a technique for growing large colonies of fibroblasts by periodically cutting away all the old coagulum surrounding the colony of cells and then building a new coagulum around them. Embryo juice is supplied, of course, as the nutrient. In this way he has obtained cell colonies 2½ cm. in diameter.³ But in

* Reported in brief in *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 369.

¹ Ephrussi, B., *Arch. anat. micr.*, 1933, **29**, 95. Fischer, A., *Cytologia*, 1930, **1**, 217. Virchows *Arch. path. Anat.*, 1930, **279**, 94. Mayer, E., *Arch. Entwicklungsmechn. Organ.*, 1933, **130**, 382; *Compt. rend. Soc. biol.*, 1935, **119**, 422. Olivo, O. M., *Arch. exp. Zellforsch.*, 1931, **11**, 272.

² Ephrussi, B., *Arch. anat. micr.*, 1933, **29**, 95. Fischer, A., *Cytologia*, 1930, **1**, 217.

³ Mayer, E., *Compt. rend. Soc. biol.*, 1935, **119**, 422.

all of this work no attention has been paid to the nutritive value of the serum that is gradually removed from the coagulum as cultivation is continued, and is present again when a new coagulum is formed. The purpose of the present investigation was to ascertain if it is not the removal of this serum, rather than the aging of the coagulum, that is responsible for the cessation of growth. Or, to express it in another way, to ascertain if the cessation of growth were not due to an inadequacy in the food supplied.

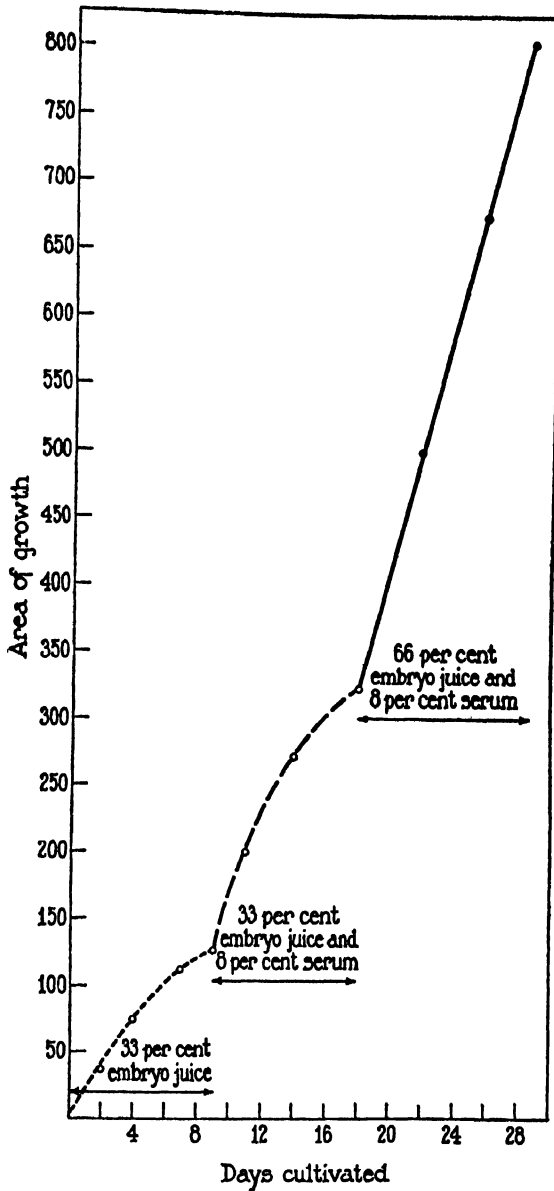
Preliminary Experiment

To test this hypothesis, two fragments of chick heart fibroblasts from a 23 year old strain were embedded in a Carrel flask in a coagulum containing 33 per cent plasma, and cultivated for 9 days in 33 per cent embryo juice.⁴ By this time their initially rapid growth had already decreased to a noticeable degree.⁵ 2 drops of chicken serum were then given every 2 days in addition to the embryo juice that had previously been supplied. Active growth was immediately resumed. However, after 9 more days of cultivation, it was evident that the rate of growth was decreasing again. Since the colonies had now become quite large it seemed that this decrease in growth might be due to an insufficient amount of food rather than to an inadequacy in the nature of the food given. Therefore, the concentration of the embryo juice in the medium was increased from 33 to 66 per cent, and the serum was given as before. Again, active growth was resumed; and this time it continued until the edge of one of the colonies reached the vertical side of the flask. A growth curve of one of these colonies showing the changes in rate of growth with each change in the medium is reproduced in Text-fig. 1.

These results indicate that two changes in procedure are required to produce continued growth of heart fibroblasts; first, that serum as well as embryo juice should be given, and second, that the embryo juice should be supplied in higher concentration than has previously been thought desirable. The experiments that follow were designed to test each of these hypotheses in as thorough a manner as possible.

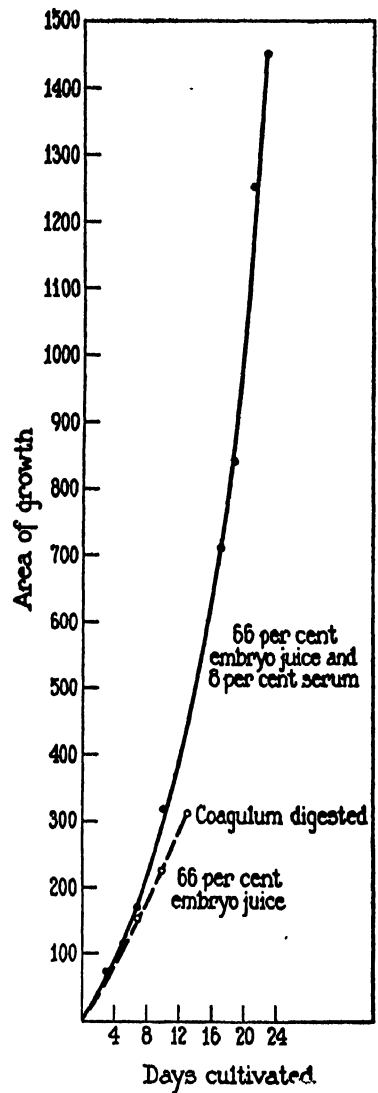
⁴ The embryo juice was prepared by extracting one volume of the latapie pulp of 9 or 10 day old embryos with 3 volumes of Tyrode's solution.

⁵ The coagulum in this experiment was made of horse plasma. This accounts for the decrease in the rate of growth sooner than it is usually observed with tissues embedded in coagula made of chicken plasma.



TEXT-FIG. 1

TEXT-FIG. 1. Experiment 15565-D. Growth curve of chick heart fibroblast culture from a 23 year old strain which was cultivated for 9 days in 33 per cent embryo juice, then for 9 days in 33 per cent embryo juice and 8 per cent chicken serum, and finally for 11 days in 66 per cent embryo juice and 8 per cent serum. Two tissues in a flask. Coagulum made of citrated horse plasma.



TEXT-FIG. 2

TEXT-FIG. 2. Experiment 15753-D. Comparison of extent and duration of growth of sister colonies of chick heart fibroblasts in their seventh passage *in vitro*, one cultivated in 66 per cent embryo juice, the other in 66 per cent embryo juice supplemented with 8 per cent chicken serum. A single fragment of tissue in each flask. Coagula made of chicken plasma.

Confirmatory Experiments

The confirmatory experiments that were made may be divided into three groups. Those in the first group were designed to test the extent and duration of growth of sister colonies of fibroblasts when one was given embryo juice alone in the nutrient fluid, the other a mixture of embryo juice and serum. 66 per cent embryo juice was supplied to both cultures in some of these experiments, 33 per cent in others. The concentration of serum was varied from 4 to 8 per cent. In the second group of experiments the extent and duration of growth of sister colonies of fibroblasts was compared when one was cultivated in 33 per cent embryo juice, the other in 66 per cent embryo juice. In some of these experiments no serum was given to either culture. In others, serum was supplied to both. The third group of experiments was designed to ascertain whether the serum was needed mainly as a nutrient or functioned merely to preserve the coagulum. In the first of these experiments embryo juice at 66 per cent concentration was given to both cultures. One of them received 4 per cent serum in addition, the other 8 per cent serum. Then sister colonies of fibroblasts were cultivated without a coagulum, one in embryo juice alone, the other in a mixture of embryo juice and serum.

Procedure Followed in the Confirmatory Experiments

All the experiments were performed first with chick heart fibroblasts from a 23 year old strain. Then a number of them were repeated with chick heart fibroblasts from a new strain that had been cultivated only six passages *in vitro* before it was taken for these experiments.⁶ Each culture was divided into two equal parts. These were cultivated in separate Carrel flasks 3½ cm. in diameter. With the exception of the few experiments in which a fluid medium was used, they were embedded in coagula 1.2 cc. in volume, containing 33 per cent plasma, and embryo juice at whatever concentration was to be used in the medium. Homologous plasma was used in some of the experiments, heterologous plasma (citrate horse or citrated irradiated cow plasma) in others. Coagulation of the citrated plasma was brought about by the addition of calcium-Ringer's solution prepared as described by Vogelaar and Erlichman.⁷ Unless otherwise specified, a single

⁶ A few experiments were also made with fresh heart tissue taken directly from the embryo. A longer time was required to obtain the results with the fresh tissue since it contained enough stored nutriment to maintain the cells for some time.

⁷ Vogelaar, J. P. M., and Erlichman, E., *Am. J. Cancer*, 1933, 18, 28.

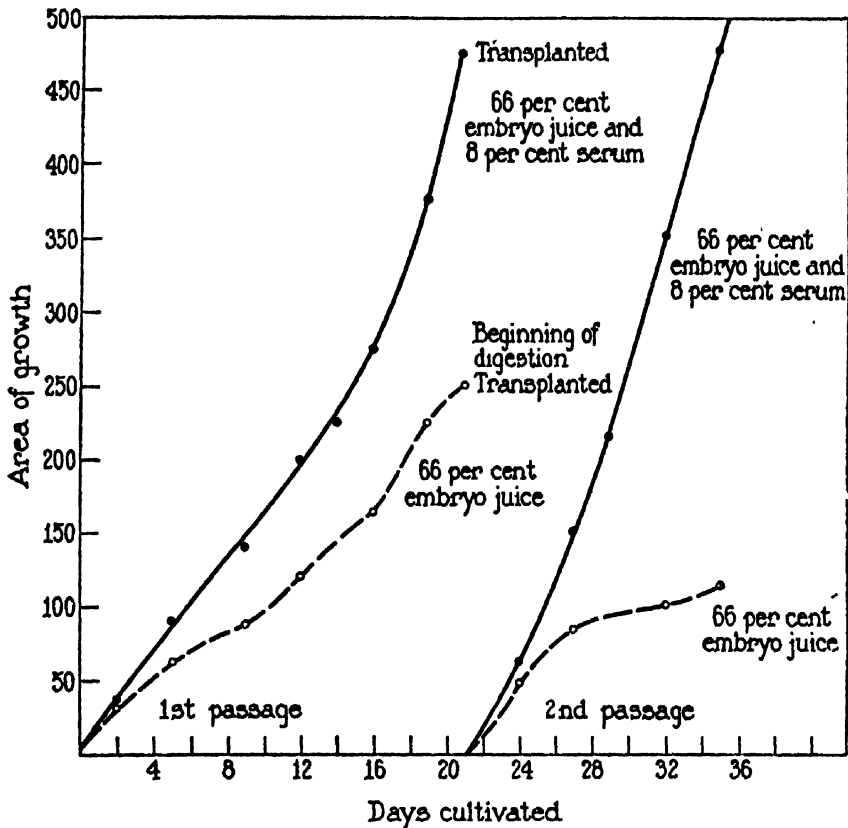
fragment of tissue was placed in each flask as near the center of the coagulum as possible. When the coagula were made with homologous plasma, no serum was given during the first week of cultivation since that already in the coagulum sufficed during this time. Phenol red at 0.005 per cent concentration was incorporated in all media to serve as an indicator of pH, and the acidity was adjusted by using a gas mixture containing 3 per cent CO₂, 21 per cent O₂, and 76 per cent N₂. Every 2 days the cultures were washed for 2 hours at 37°C. with 1½ cc. of their respective media. Then this wash fluid was withdrawn and the cultures were returned to the incubator. Whenever the cell colonies became very large, new medium was given every 24 hours instead of every 48 hours, and 0.5 cc. of medium was placed on the culture after the wash fluid had been withdrawn. The embryo juice was generally made by extracting for ½ hour one volume of pulp from 9 or 10 day old chick embryos with three volumes of Tyrode's solution. In some instances the embryo juice was made with glucosol solution. Then Tyrode's solution containing twice the usual amount of bicarbonate was used to complete the media. The serum was taken in each instance from adult cocks.

Comparison of Growth in Embryo Juice with and without Serum

In the experiments of group I, in which the tissues were embedded in homologous plasma and embryo juice was supplied at 66 per cent concentration, the sister colonies, *i.e.*, those given embryo juice alone and those given serum with the embryo juice, proliferated very rapidly and at approximately the same rate for 11 or 12 days. Then the colonies that were being cultivated in embryo juice alone suddenly liquefied the coagula. Those that were given serum with the embryo juice continued their active proliferation until the entire coagulum was covered with tissue (Text-fig. 2). Thus, colonies that were 3 to 3½ cm. in diameter were obtained. The time required for the cells to cover the coagulum varied with the original condition of the tissue. The new strain of fibroblasts that had been cultivated only six passages *in vitro* before it was taken for this experiment proliferated so rapidly that the cells from a single fragment of tissue covered the coagulum in 17 days. Single fragments of tissue from the 23 year old strain required from 25 to 36 days in the different experiments.

In an attempt to circumvent digestion of the coagulum by the tissue cultivated in embryo juice alone the experiments described above were repeated, using heterologous plasma in making the coagula. Under these conditions liquefaction of the clot was delayed but not prevented. However, before the coagula were digested a marked difference in the

rate of growth of the colonies cultivated with and without serum was noted. Those that received serum with the embryo juice increased in area and also in density more rapidly than those cultivated in embryo juice alone. While the colonies cultivated in embryo juice alone proliferated mainly at the periphery of the culture, those culti-



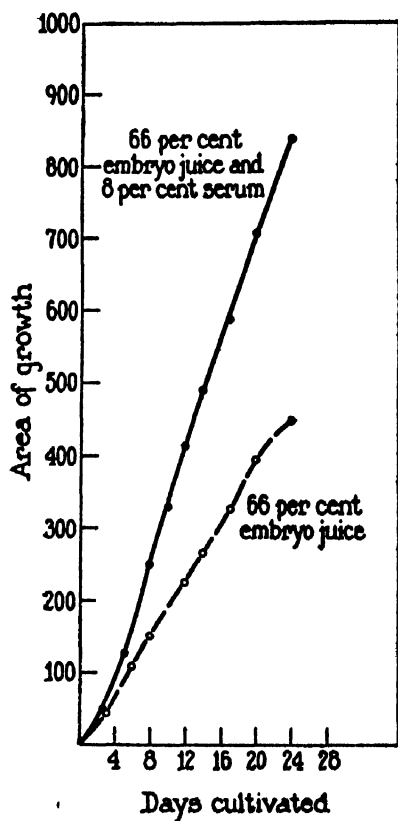
TEXT-FIG. 3. Experiment 15477-D. Comparison of the growth of sister colonies of chick heart fibroblasts from a 23 year old strain embedded in coagula made of irradiated, citrated cow plasma, one cultivated in 66 per cent embryo juice, the other in 66 per cent embryo juice and 8 per cent chicken serum. Two fragments in a flask. The tissues were transplanted into new coagula on the 21st day because those in embryo juice alone were beginning to digest the coagulum.

vated in the mixture of serum and embryo juice proliferated to a considerable extent at the center as well, and formed colonies containing many layers of cells. After 2 or 3 weeks of cultivation the colonies that were given embryo juice alone liquefied the coagula. Those cultivated in embryo juice and serum proliferated as long as

cultivation was continued. Curves illustrating the difference in rate of growth in one of these experiments, in which the tissues were transplanted when those in embryo juice alone began to digest the coagulum, are reproduced in Text-fig. 3.

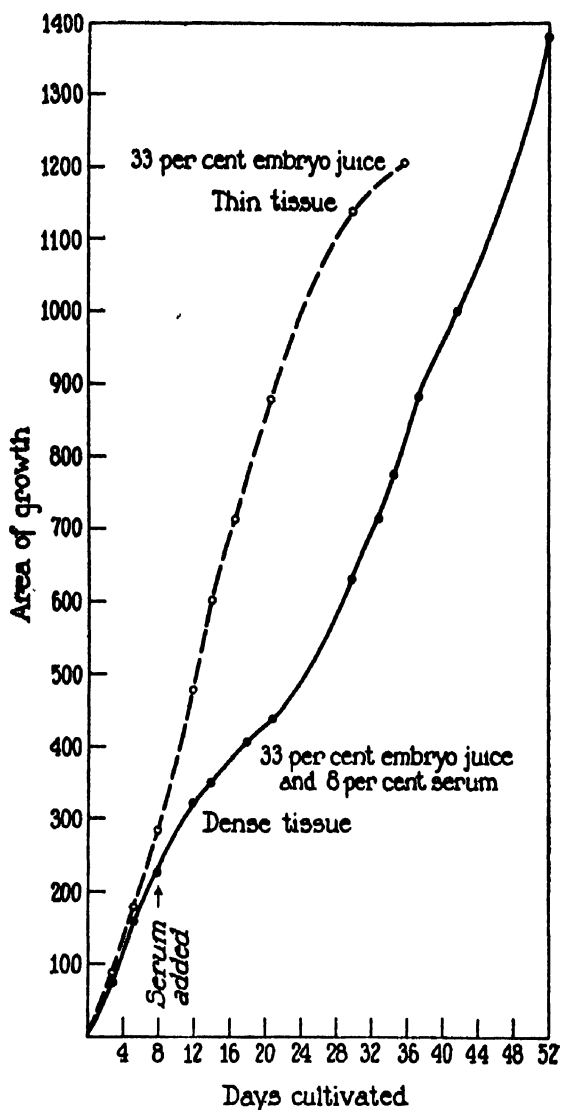
To see if the difference in the rate of growth observed in the heterologous plasma could be observed in homologous plasma also, the experiments were made once more in coagula of homologous plasma. But this time, the clots were washed as soon as coagulation had occurred so as to remove a considerable part of the serum they contained. Under these conditions the same difference in growth was observed as had been noted in the heterologous plasma (Text-fig. 4).

The results obtained in 33 per cent embryo juice differed considerably from those just described. The control colonies, *i.e.*, those given embryo juice alone as nutrient fluid, behaved differently in different experiments. Usually they proliferated rapidly at first, then more slowly, and finally not at all. Soon after their proliferation ceased, the cells degenerated. The growth curve of these colonies was the typical S-shaped curve that has frequently been described.² However, in a few instances, growth continued 28 days or longer (Text-figs. 5 and 6). Therefore, colonies of large area were formed, but the cells in those colonies were scattered and the tissue was exceedingly thin. It was obvious, moreover, that the cells within these colonies were gradually digesting the coagula, and obtaining additional nutrient thereby. The coagula became thinner and thinner, and in certain instances appeared to be consumed in that region that was covered with cells. When serum was given with the embryo juice, the coagula retained their original thickness for a long time. Moreover, the tissue formed in the mixture of embryo juice and serum was always more dense, and was composed of a larger number of cell layers than was that formed by the sister colony cultivated in embryo juice without serum (Figs. 1, 2, and 3). When 8 per cent serum was given with 33 per cent embryo juice the cells became quite granular, and the colony cultivated in embryo juice and serum increased in area less rapidly than the sister colony that was given only embryo juice in the nutrient fluid. But since the growth continued for a longer time when serum was present, the colonies in the mixture of embryo juice and serum eventually outgrew those given embryo juice alone (Text-



TEXT-FIG. 4

TEXT-FIG. 4. Experiment 15747-D. Comparison of the growth of sister colonies of chick heart fibroblasts from a 23 year old strain, one cultivated in 66 per cent embryo juice, the other in 66 per cent embryo juice and 8 per cent serum. Coagula made of chicken plasma and then washed to remove a part of the serum.



TEXT-FIG. 5

TEXT-FIG. 5. Experiment 15826-D. Comparison of the growth of sister colonies of chick heart fibroblasts from a 23 year old strain, one cultivated in 33 per cent embryo juice, the other in 33 per cent embryo juice supplemented with 8 per cent serum. Coagula made of chicken plasma. One fragment of tissue in a flask. The colony in embryo juice alone consisted of a very thin growth of rather scattered cells. The other formed a dense tissue. The former derived additional nourishment by slow digestion of the coagulum.

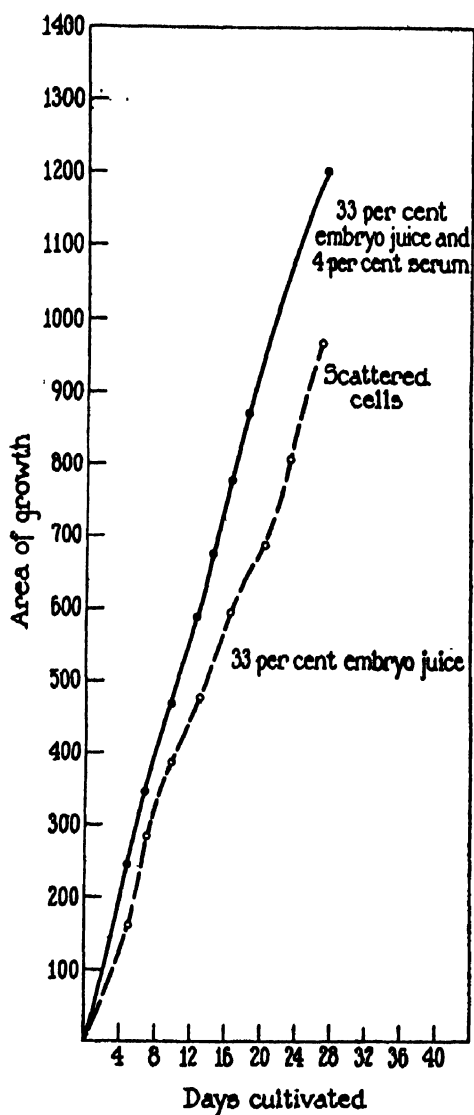
fig. 5). When 4 per cent serum was given with 33 per cent embryo juice, the cells remained in excellent condition; and the colony which received the serum increased in area and also in thickness more rapidly than did the sister colony that was cultivated in embryo juice without serum (Text-fig. 6). But growth in this medium was not continuous. After colonies 2 or $2\frac{1}{2}$ cm. in diameter were obtained, proliferation ceased. Yet the cells cultivated in the mixture of 33 per cent embryo juice and 4 per cent serum did not degenerate, as those in embryo juice alone did. Some of them were kept under cultivation for 2 and 3 months. Throughout this time they seemed to be in good condition. Then, when a small fragment of the tissue was transplanted, they proliferated again. It seems, therefore, that an equilibrium is reached in this medium in which the food supplied is sufficient to maintain a large colony of cells, but is not sufficient to promote further proliferation.

Comparison of Growth in 33 Per Cent and 66 Per Cent Embryo Juice, with Serum, and without Serum

In all those experiments in which the growth in 66 per cent embryo juice was compared with that in 33 per cent embryo juice, a much more rapid growth was always obtained at the higher concentration. When no serum was supplied with the embryo juice the colonies that were given embryo juice at 66 per cent concentration always liquefied the coagulum. Those cultivated in 33 per cent embryo juice proliferated actively at first then more slowly, and then as a rule degenerated without digesting the coagulum. When serum was given with the embryo juice the colonies that received embryo juice at 66 per cent concentration proliferated until the entire coagulum was covered with tissue. Those given embryo juice at 33 per cent concentration proliferated for a very long time but never filled the flask (Text-fig. 7).

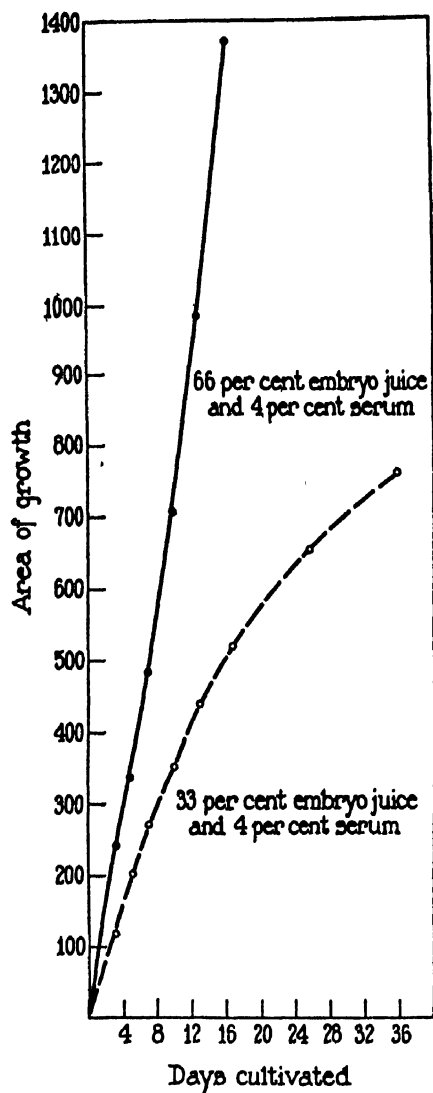
Cultivation with and without a Coagulum in Media Containing Different Amounts of Serum

So much has been said concerning the probable effect of changes in the physical structure of the coagulum on growth that it seemed advisable to establish, if possible, whether the growth-promoting effect



TEXT-FIG. 6

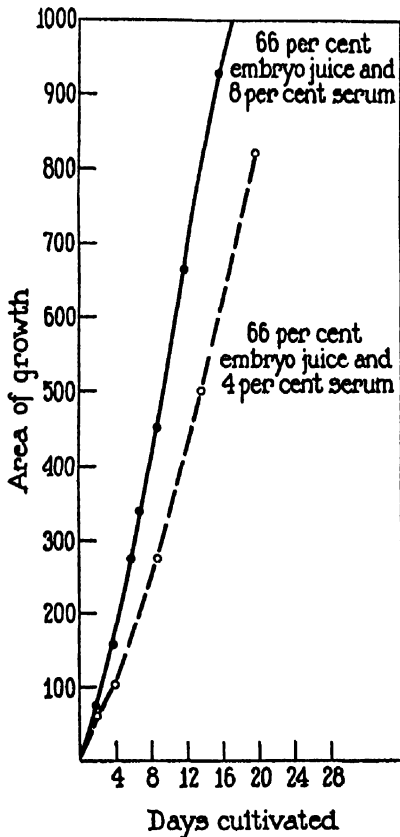
TEXT-FIG. 6. Experiment 15811-D₁₄₃. Growth curves of sister colonies of chick heart fibroblasts in their seventh passage *in vitro*, one of which was cultivated in 33 per cent embryo juice, the other in 33 per cent embryo juice supplemented with 4 per cent serum. A single fragment of tissue in each flask. Coagula made of chicken plasma. The tissue in embryo juice alone was not as dense as the other, and it digested its coagula at a very slow rate.



TEXT-FIG. 7

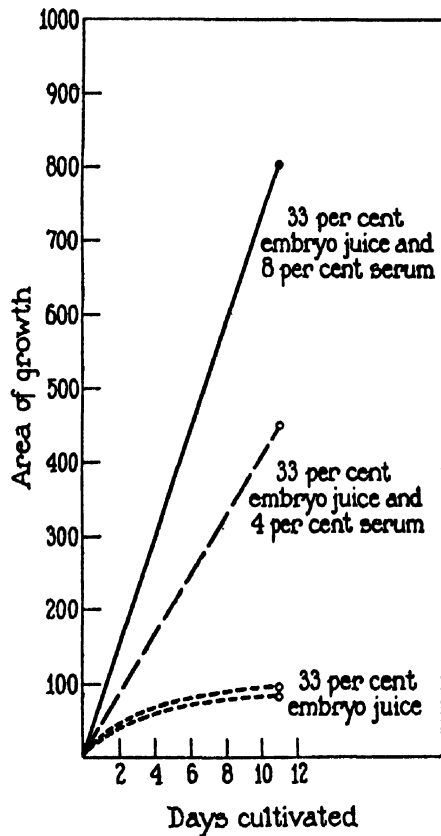
TEXT-FIG. 7. Experiment 17811-D₇₄₃. Growth curves of sister colonies of chick heart fibroblasts in their seventh passage *in vitro*, one of which was cultivated in 33 per cent embryo juice supplemented with 4 per cent serum, the other in 66 per cent embryo juice supplemented with the same amount of serum. A single tissue in each flask. Coagula made of chicken plasma.

of the serum, described under the experiments of group I, was the direct effect of nutrients in the serum, or the indirect effect of its



TEXT-FIG. 8

TEXT-FIG. 8. Experiment 15911-D. Growth curves of sister colonies of chick heart fibroblasts from a 23 year old strain, one cultivated in 66 per cent embryo juice and 4 per cent serum, the other in 66 per cent embryo juice and 8 per cent serum.



TEXT-FIG. 9

TEXT-FIG. 9. Experiment 15818-D. Areas attained in 11 days by four sister colonies of chick-heart fibroblasts in their seventh passage *in vitro*, when cultivated in fluid medium, two of them in 33 per cent embryo juice, one in 33 per cent embryo juice and 4 per cent serum, and one in 33 per cent embryo juice and 8 per cent serum.

preservative action on the coagulum. In the first experiment made to throw some light on this question, sister colonies of fibroblasts were cultivated in 66 per cent embryo juice. One of them was given 4

per cent serum in addition, the other 8 per cent serum. The coagulum was preserved in both instances, but greater proliferation was obtained with 8 per cent serum (Text-fig. 8). In the second experiment the tissues were cultivated in fluid media, in embryo juice alone, and in a mixture of embryo juice and serum. Cells from the colonies cultivated in embryo juice alone migrated out onto the glass, but the tissue that was formed broke up almost immediately into islands of a few cells each, then into isolated cells which soon died (Figs. 4 and 5). Those cultivated in the mixture of embryo juice and serum proliferated much more actively. The cells migrated out onto the glass and formed a tissue of connected cells which showed no tendency to break up into isolated cells. Then groups of cells migrated further out, forming islands of tissue that covered the entire flask^{8, 9} (Figs. 6 and 7). In these experiments as in those just described, a marked difference in the rate of growth was noted according to the amount of serum supplied, that in the medium containing 8 per cent serum being greater than that in the medium containing only 4 per cent (Text-fig. 9).

DISCUSSION

It is evident from the results described above that the primary cause of the discontinuance of growth of heart fibroblasts when they are cultivated in a plasma coagulum with embryo juice as nutrient fluid is the removal of serum from the coagulum. When serum as well as embryo juice is supplied in the nutrient fluid the cells proliferate more actively and for a longer time than they do in embryo juice alone. Yet active growth does not continue unless the embryo juice is given

⁸ A strikingly different result was obtained with fresh heart tissue in fluid medium. Fairly large colonies of fibroblasts were obtained in embryo juice alone, and these adhered to the flask for a much longer time than did the tissue cells from the pure strain. No attempt has been made to ascertain for how long a time the tissue could be cultivated under these conditions.

⁹ Fibroblasts have been cultivated by des Ligneris for several months in hanging drop cultures in a medium prepared by extracting embryo pulp with serum. No attempt was made in that work to ascertain the relative part played by the two constituents of the medium. The precipitate that was formed in the medium on standing also appeared in this work and eventually obscured the outline of the cells (des Ligneris, M. J. A., *Arch. exp. Zellforsch.*, 1936, 18, 442).

at higher concentration than that usually supplied. The question of promoting continuous growth seems, therefore, to be mainly a question of supplying adequate nourishment. A means must be found, of course, of bringing the nourishment into intimate contact with the cells, and of removing the waste products. The efficiency with which this is done assumes ever greater importance as the mass of the tissue increases. The serum functions in two ways. It supplies nutriment that is needed by the cells and prevents digestion of the coagulum. Sometimes in its absence the cells digest the coagulum at a very slow rate and obtain thereby enough nutriment to enable them to live for a considerable period of time, but their proliferation under these conditions is never as rapid as it is when serum is present.

That serum is a valuable nutrient for fibroblasts has been known for a long time. Carrel in his first experiments with tissues cultivated outside the body maintained heart and other embryonic tissues in plasma and serum for 2 months or longer.¹⁰ Olivo in 1931 cultivated a fragment of embryonic heart for 6 months in diluted plasma and serum;¹¹ and Parker maintained fibroblasts from various sources in diluted serum for periods extending from 92 days to an entire year.¹² But in all of these experiments growth was exceedingly slow. In Carrel's experiments the fragments grew smaller and smaller, a part of the tissue being lost at each transfer. Olivo reports that in 6 months cultivation the fragment only doubled its size; and Parker states that while serum promotes a slow growth, the first effect of the serum is invariably injurious. The degree of injury, and also the ability of the cells to utilize the serum and proliferate in it varies with the different strains, *i.e.*, according to the source from which they are derived. Fibroblasts from heart utilized the serum less readily than did the other strains and suffered greater injury at first.¹² Moreover, in Parker's experiments the muscle fibroblasts which he cultivated for an entire year grew so slowly that it was not necessary to transfer them to a new flask throughout that time. The marginal cells of such

¹⁰ Carrel, A., *J. Am. Med. Assn.*, 1911, 57, 1611; *J. Exp. Med.*, 1912, 15, 516.

¹¹ Olivo, O. M., *Arch. exp. Zellforsch.*, 1931, 11, 272.

¹² Parker, R. C., *J. Exp. Med.*, 1933, 58, 97; 1936, 64, 121.

cultures invariably died, although new ones came from the central fragment at periodic intervals.

Earle has reported that horse serum is a satisfactory medium for mammary carcinoma and normal fibroblasts taken from the subcutaneous tissues of the rat, and that the growth of these tissues in horse serum is increased by the addition of embryo juice. He also records in passing that the fibroblasts grew more actively in this mixture than in any he had tried.¹³ Lewis, and Gey and Gey have found mixtures of human cord serum, rat serum, and embryo juice the most suitable medium for sustaining growth of various tissues in their roller tube technique.¹⁴ Yet the idea that heart fibroblasts require only embryo juice as a nutrient still persists. Moreover, none of these workers have attempted to show by comparative tests the part played by the serum in producing and maintaining growth. Therefore, although some of the results reported¹⁵ here are implied in some of this work, it has seemed advisable to report these comparative experiments which demonstrate that even that strain of fibroblasts which utilizes serum the least readily of all the fibroblasts, when serum alone is given, actually requires serum for its growth.

No effort has been made in this work to determine the optimum ratio of serum to embryo juice or the exact composition of a medium that would promote maximum growth. It is evident, nevertheless, that the concentration of serum should be varied with that of the embryo juice.

It is probably important to note that these experiments do not necessarily invalidate those reported many years ago by Carrel and his

¹³ Earle, W. R., *Am. J. Cancer*, 1935, **24**, 567.

¹⁴ Lewis, W. H., *Carnegie Institution of Washington, Pub. No. 150, Contrib. Embryol.*, 1935. Gey, G. O., and Gey, M. K., *Am. J. Cancer*, 1936, **27**, 45.

¹⁵ The experiments reported here were actually performed in 1933 and 1934. Pressure of other work being done at the time prevented their immediate publication. Then this other work which implied these results appeared, so publication did not seem essential. Yet because these experiments show better than others the real part played by the serum, and because of the persistence of the idea that serum inhibits the growth of heart fibroblasts, it has seemed advisable to present them at this time.

coworkers¹⁶ on the inhibiting effect of serum when it is used with embryo juice. In those old experiments serum was always used at high concentration, usually as $\frac{1}{2}$ or $\frac{2}{3}$ of the medium; while here it was used at 8 per cent concentration or less.¹⁷ In a few of these experiments, in which the embryo juice was used at 33 per cent concentration and serum was given at 8 per cent concentration,¹⁷ the effect which serum had in restricting the area of migration was very evident. Moreover, the cells became very fatty and granular when the ratio of serum to embryo juice was too high. Since serum is antitryptic in its action, and since the embryo juice is enzymatic, it may well be that a high concentration of serum prevents growth by inhibiting enzymatic action, while a low concentration promotes it. It is also important to note that these experiments do not necessarily mean that it is impossible to prepare from embryos alone a medium that will meet all the nutritive requirements of the fibroblast; but until the continuous proliferation of fibroblasts in embryo juice alone has been found possible, it will be well to consider serum as an essential part of the medium.

The findings reported here apply only to heart fibroblasts since it is only heart fibroblasts that have been used in this work. In all probability the same will be found true of fibroblasts from other sources. However, it is not advisable to assume that such is the case for all fibroblasts until it has been submitted to experimental test, for as Parker has shown, fibroblasts of different origins have different nutritional requirements.

SUMMARY

Experiments designed to ascertain the reason for the cessation of growth of heart fibroblasts when they are cultivated in a plasma coagulum with embryo juice as nutrient fluid have shown that it is due, first, to the gradual removal of serum from the coagulum, and

¹⁶ Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, **34**, 317, 599; 1922, **35**, 17, 647; **36**, 399; 1923, **37**, 759. Baker, L. E., and Carrel, A., *J. Exp. Med.*, 1925, **42**, 143; 1927, **45**, 305.

¹⁷ Actually the concentration of serum was somewhat higher than this since there was also some serum in the coagulum.

second, to an insufficient supply of embryo juice. In a medium containing embryo extract at 66 per cent concentration and serum at 8 per cent concentration, growth continued until the entire coagulum in a $3\frac{1}{2}$ cm. flask was covered with tissue. The serum is needed to furnish additional nutriment, and also to prevent digestion of the coagulum.

EXPLANATION OF PLATE 37

FIGS. 1 and 2. Photographs showing the relative density of sister colonies of fibroblasts cultivated in embryo juice with and without serum. Fig. 1, fibroblasts near the periphery of a colony, cultivated for 36 days in 33 per cent embryo juice; Fig. 2, fibroblasts in a corresponding area of a sister colony, cultivated for 36 days in 33 per cent embryo juice and 4 per cent serum. $\times 115$.

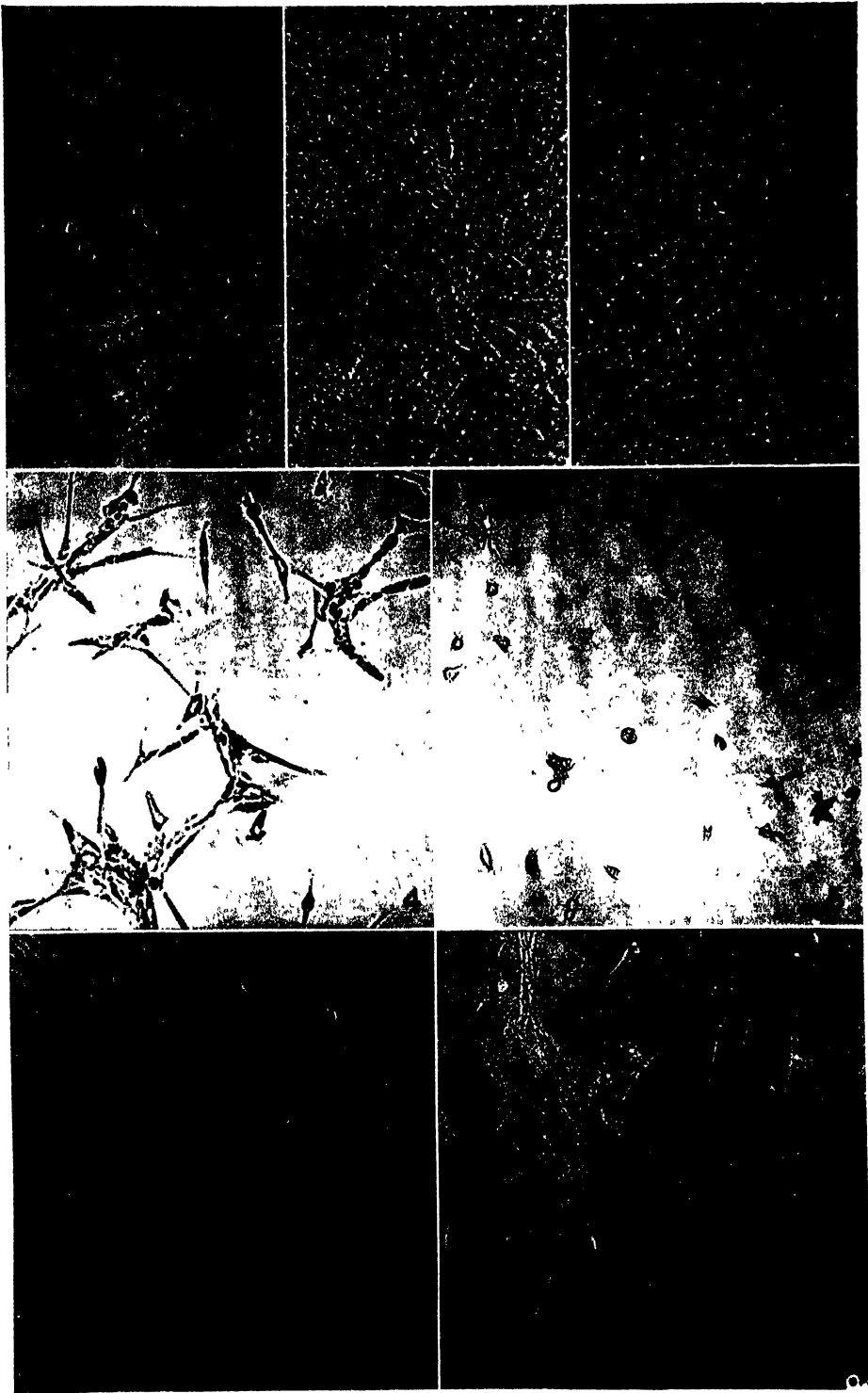
FIG. 3. Same colony as shown in Fig. 2. An area near the center of the colony. $\times 115$.

FIG. 4. Cells that have migrated out onto the glass from colonies of chick heart fibroblasts from a 23 year old strain cultivated for 5 days in 33 per cent embryo juice. $\times 115$.

FIG. 5. The same cells as shown in Fig. 4 after 16 days cultivation in 33 per cent embryo juice. $\times 115$.

FIG. 6. Cells from a sister colony to that shown in Fig. 5 cultivated for 16 days in a mixture of 33 per cent embryo juice and 8 per cent serum. Cells near the center of the colony. $\times 115$.

FIG. 7. Cells at the periphery of the colony shown in Fig. 6, after 16 days cultivation in 33 per cent embryo juice and 8 per cent serum. Such a network of cells covered the entire flask. $\times 115$.



(Baker: Cessation of growth of fibroblasts)

ARTIFICIAL MAINTENANCE MEDIA FOR CELL AND ORGAN CULTIVATION

II. THE CULTIVATION OF ORGANS IN ARTIFICIAL MEDIA*

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PLATES 1 TO 3

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The development of the Carrel-Lindbergh technique (1) for the cultivation of whole organs has opened up many possibilities in the field of biological research. But to realize all these possibilities it is necessary to have artificial media that can be used in the place of blood and serum, to nourish the organs after they have been removed from the body. If such media could be found, the cost of experimentation would be reduced, studies on the behavior of human organs under various pathological conditions would become possible, and the organs of animals too small to yield sufficient serum could be utilized. Moreover, many kinds of experimental work, difficult to carry on when serum has to be used as a nutrient, would be facilitated; as, for instance, the isolation of substances of protein nature secreted by the organs, and the study of the effect of the complete absence in the medium of some of the substance normally present in serum.

Artificial media, developed with this end in view, by means of the tissue culture technique, were described in the first article of this series (2). Experiments in which these media were tested for their power to sustain the life of a pure strain of fibroblasts *in vitro* were also reported (2). The purpose of the present communication is to describe the results obtained on using these media for the cultivation of whole organs¹ in the Lindbergh apparatus.

In a number of these experiments the organs were kept under cultivation for only 3 or 4 days, *i.e.*, no longer than some of them survive when perfused with Tyrode's solution. Yet even within these brief periods marked changes in some of the glands were noted, and differences in the effects of the various

* Reported in brief by Carrel and Lindbergh (3).

¹ The cultivation of these organs was carried out by a number of members of this department. Miss Irene McFaul has performed the operations in a large number of the experiments. Dr. Raymond Parker and Dr. Albert Ebeling have performed the operations for some others.

media observed. In other experiments the cultivation was continued for periods varying from 10 days to several weeks.

Methods

No description of the cultivation technique will be given here, since that has already been described (3). When the experiments were terminated the cultivated organs were separated from the arteries and connective tissue and fixed in Zenker-formol, or in Bouin's picro-formol solution. They were then sectioned and stained with hematoxylin-eosin, and also, in some instances by Goldner's (4) modification of the Masson trichrome technique. When glands that occur in pairs in the animal were cultivated, one of them was removed at the time of operation and was fixed immediately in the same manner as the cultivated gland, so that a comparison of the cultivated and the uncultivated, or control gland, could be made, and the changes resulting from the cultivation determined.

EXPERIMENTAL

Results with Medium I.—

The simplest medium (medium I), as described in the first article of this series, was made up of a digest of whole blood (2), 2 per cent serum, and Tyrode's solution. It was modified for the experiments described here by the addition of tryptophane (5 mg. per cent) and potassium iodide (0.13 mg. per cent). The glucose concentration was increased to 300 mg. per cent, and phenol red (0.05 per cent) was added to serve as an indicator of pH.

A cat ovary that was cultivated in this medium for 4 days was found to be indistinguishable from the control ovary taken from the same animal and fixed without cultivation. The cells within it appeared to be entirely normal and well nourished. Its ova, in their different stages of maturation, were well preserved, and a corpus luteum was in excellent condition. Photographs of portions of this gland are shown in Figs. 1 and 2. A guinea pig heart cultivated in this medium, with the serum concentration increased to 5 per cent, pulsated for 3 days. The heart muscle and the tissue of the lung that was cultivated with it were both found to be in excellent condition. A thyroid gland cultivated for 6 days was likewise well preserved. Comparison of sections of this gland with those of the control gland showed that the epithelium had become lower during the cultivation, and the colloid less vacuolated. A few dead cells coming from a small area that degenerated will be seen in between some of the follicles, Fig. 3.

Results with Medium II.—

The second medium, as reported in the experiments on fibroblasts (2), consisted of blood digest, cysteine hydrochloride, insulin, hemin, thyroxin, vitamins A, B₁, B₂, C, and D, glutathione, potassium iodide, and Tyrode's solution made with 300 mg. per cent glucose. This was modified for the organ culture experiments by the addition of tryptophane, 5 to 10 mg. per cent, and in some instances by the addition of a small

amount of Witte's peptone, enough to give 7.4 mg. per cent nitrogen. No serum was used here except that small amount, 0.05 per cent, required as a solvent for vitamins A and D.

A considerable number of cat thyroids were cultivated in this medium, the perfusion being continued for 6 days in each case. Consistently good results were obtained. In each case the glands seemed to be normal² and well nourished. Comparison of the cultivated glands with the uncultivated controls showed that the epithelium had become lower and the colloid less vacuolated during the cultivation. An illustration from one of these experiments will be found in Fig. 4.

This same medium with 2 per cent serum, or 0.1 per cent casein, added, was used by Bauer in perfusion experiments undertaken in this laboratory with guinea pig heart, lung, liver, kidney, suprarenal gland, and other organs. These experiments have been reported elsewhere (5). When guinea pig serum was not available, cat serum heated for one hour at 56°C. was used instead. No harmful effect of the foreign serum was noted. The serum was incorporated in the medium in these experiments partly because of its added nutritive value, and partly to insure smooth action of the valves of the pumps, some of which were not sufficiently well ground to function smoothly in a serumless medium. The addition of a small amount of casein was found later to have the same effect on the valves.³

Results with Medium III.—

Medium III, as used in the experiments with fibroblasts, had the following composition:

	<i>per 100 cc.</i>		<i>per 100 cc.</i>
Whole blood digest, in quantity to give.....	60 mg. N	Hemin.....	0.004 mg.
Tryptophane.....	5 to 10 mg.	Vitamin A dissolved in serum (0.05 cc.). ⁴	The vitamin A concentrate used here contained 1 unit vitamin D for each 5 units A ⁵
Cysteine hydrochloride.....	9.0 mg.		100 units
Insulin.....	0.1 unit		
Thyroxin.....	0.001 mg.		

² Detailed cytological studies such as were made by Okkels on the cultivated thyroid (*J. Exp. Med.*, 1937, **66**, 297) have not been possible in this work. The term "normal" as here used refers to the structure as revealed in sections stained with hematoxylin and eosin.

³ Casein, when tested for its toxic effect on fibroblasts in tissue culture, was found to be injurious at twice this concentration. However, at this concentration it was apparently not harmful during a 4 week experiment.

⁴ Cat serum was used as the solvent for vitamin A even when the medium was used for the cultivation of the human thyroid.

⁵ The amount of vitamin D taken up by the serum has not been determined.

	<i>per 100 cc.</i>		<i>per 100 cc.</i>
Vitamin B ₁	0.1 gamma	Urea.....	2.4 mg.
Vitamin B ₂	3.4 gammas	Glycerine.....	0.2 cc.
Ascorbic acid.....	0.3 mg.	Thymus nucleic acid.....	20.0 mg.
Glutathione.....	1.2 mg.	Antuitrin.....	0.2 cc.
Glucose.....	300.0 mg.	Adrenalin chloride.....	0.1 cc.
Potassium iodide.....	0.13 mg.	Eschatin, suprarenal cortex	
Phenol red.....	5.0 mg.	hormone.....	0.1 cc.
Witte's peptone, to		Pitressin, pituitary hormone...	0.1 cc.
supply.....	6.0 to 7.4 mg. N	Casein ⁶	0.1 gm.
Sodium glycerophosphate.....	57.3 mg.		

This medium, prepared according to the above formula, and with certain modifications as will be noted under the individual experiments, has been used for the cultivation of a large number of glands including the prolonged cultivation of the human thyroid. The uterus and ovaries of a cat which were perfused for 4 days were found in excellent condition. A portion of the uterine mucosa from one of these experiments is shown in Fig. 5. A uterus cultivated for 15 days in this medium with the eschatin, pitressin, and adrenalin reduced to 1/5 the concentrations given above, was not so well preserved.⁷ Some areas within the gland had degenerated, while others in close juxtaposition were still in good condition. A portion of the uterine mucosa is shown in Fig. 6. All the ova in an ovary that was cultivated for 18 days in the original medium to which a suspension of lecithin and cholesterol was added were degenerated. But the cells of the follicles and much of the interstitial tissue were still in good condition (Fig. 7). Another ovary perfused for 39 days with the original medium was in approximately the same condition but had somewhat larger areas of degeneration. It is probable that there was a stoppage of circulation to parts of these glands, due either to particles of the suspension or to air emboli.

A cat testicle cultivated in this medium for 4 days showed some multinucleated giant cells within the tubules, but the epididymis was well preserved (Fig. 8). As the formation of multinucleated cells has been noted also in experiments in which testicles were cultivated in 40 per cent serum, it would seem that this change is due to some condition attending the cultivation, rather than to the use of the artificial medium.

Kidneys and suprarenal glands cultivated in this medium behaved in the same way as those that were cultivated in serum. Considerable portions of the gland degenerated. It is believed that this degeneration is

⁶ Harris purified casein free from vitamins A and B.

⁷ In this particular experiment the medium became slightly hypertonic during the cultivation because of incomplete saturation of the incoming air with water.

brought about by an insufficient supply of oxygen. Results that were somewhat better, but still not entirely satisfactory, were obtained by carrying out the cultivation at room temperature. The medium contains a little more oxygen under these conditions, and the metabolism of the gland is lower. Photographs of the medulla of a suprarenal cultivated for 3 days at room temperature, and of the pelvis of a kidney cultivated for 5 days, are shown in Figs. 9 and 10. In the experiment with the kidney the medium contained no serum, and no vitamin A or D.

Cat thyroids cultivated for 4 days in this medium presented a very different appearance from those cultivated in the first two media described. The cells were large, the epithelium high, and the colloid showed a considerable degree of vacuolation. They seemed to have been well nourished and to have secreted actively. A comparison of the cultivated glands with the uncultivated controls showed that the glands had become more active during cultivation. A cat thyroid cultivated for 11 days in this medium, with the adrenalin, eschatin, and pitressin reduced to one-fifth the concentration noted above, was also in excellent condition (Fig. 11). The epithelium was higher than that of the control gland, and the colloid more vacuolated. The cells were large, and the whole gland presented the appearance of being well nourished. Another thyroid cultivated for 15 days was almost completely degenerated, due to an accidental blocking of the circulation, but the parathyroid cultivated with it was alive and in good condition (Fig. 12). Thyroids perfused for 4 days in the same medium but with the serum and vitamins A and D omitted, presented the same appearance as those cultivated for 4 days in the complete medium (Fig. 13). The parathyroids were also in good condition, but showed considerably more vacuolation than did the uncultivated controls (Fig. 14). Essentially the same results were obtained in another 4 day experiment in a medium in which amino acids, obtained by hydrolyzing casein,⁸ replaced the blood digest and the Witte's peptone, and in which the vitamins, A and D, were also omitted. A human thyroid cultivated in a medium in which amino acids replaced the blood digest and peptone, but which contained vitamins A and D, was still alive after 10 days' cultivation (Fig. 15) but was quite soft and edematous.⁹

This human thyroid and others submitted to cultivation as described below, were obtained through the courtesy of Dr. Foot of the New York

⁸ Obtained from Mead Johnson and Company. Laboratory product No. 894.

⁹ Experiments on fibroblasts in tissue culture have shown the amino acid medium to be much inferior to that containing the blood digest.

Hospital. They consisted of those parts of the thyroid that were removed at operation from 12 patients suffering from Graves' disease, from 2 with nodular goiters, and 3 with adenomas. These glands were cultivated in medium III and various modifications of it for periods ranging from 5 to 35 days, with one experiment lasting 47 and another 62 days. Biopsies were made at frequent intervals, so that it was possible to follow changes occurring during the cultivation. Since previous experiments with cat thyroids had indicated that medium III stimulated the thyroid to rather great activity, changes in its hormone content were made from experiment to experiment in the hope of finding a medium that would maintain the gland more or less in its original condition. Thus, about one-third of the glands were cultivated in the medium, as described above. Another third were cultivated in the same medium with the adrenalin, eschatin, and pitressin reduced to one-fifth the concentrations originally used; and another third in a medium containing theelin (0.4 gammas per cent), adrenalin at one-fifth the original concentration, and with the thyroxin concentration increased about 50 per cent above that previously used.¹⁰ In a number of these experiments nucleic acid was left out of the medium since some previous experiments with tissue cultures indicated that it might not be needed. Whenever the vascular system in the specimen indicated that it might be possible to establish two separate systems of circulation, the tissue was divided and cultivated in two separate pumps, in media designed for comparison, as for instance, with and without adrenalin, or with and without added insulin. But in only a few instances was it possible to make such experiments and to carry both specimens for a sufficient length of time to obtain the information sought. Nevertheless, some valuable clues as to the effect of some of the constituents of the medium were gleaned by studying all the data, even though they were on glands taken from different individuals. The conclusions drawn are being reported in a joint paper with Dr. Foot who made a careful analysis of the effects of cultivation and attempted to correlate them with the variations in composition of the media, and also with the condition of the patient from whom the gland was taken. It will suffice for this report to note some of the more obvious changes that occurred, and to discuss the results from the standpoint of the nutritive efficiency of the media.

In most of these experiments there appeared sooner or later some areas in which the tissue had degenerated. In close proximity to these degenerated areas other tissue was found that was in excellent condition. It

¹⁰ The thyroxin concentration was increased in this medium because it was originally designed for use with other glands.

was obvious, therefore, that the degeneration was caused by lack of sufficient circulation in certain portions of the gland, and not by deficiency in the nutritional properties of the medium. These degenerated spots were very limited in area during the first 2 weeks of cultivation. After 4 or 5 weeks of cultivation they comprised a considerable portion of the gland, sometimes leaving only small islands of tissue that were still alive. Any one of a number of causes may have been responsible for this condition. In each experiment with the human thyroid the gland was dipped for a few seconds in Dakin's solution to insure its sterility. This brought about surface degeneration. Fragments coming from the degenerated tissue may have blocked circulation to other parts of the gland. The biopsies that were made may also have interrupted circulation to portions of the gland. Then, too, because the mass of tissue that was being cultivated was quite large, a frequent change of medium was required.¹¹ This was accomplished by transferring the organ to a new pump containing the new medium, thus increasing the possibilities for introduction of air emboli. And once degeneration occurs in any portion of the gland the possibility of the degenerating tissue clogging the capillaries to another part is increased. It seems probable also that the vascular system may have broken down eventually, due to inadequate nourishment in the artificial medium, or to its having been submitted to too great a physical strain in the apparatus. Taking all these factors into consideration, it is not surprising that localized degeneration occurred. The important fact to note is that the gland as a whole was in excellent condition in most instances after 9 or 10 days' cultivation; a considerable portion, as judged by the biopsy specimens, was still viable and in good condition after 3 weeks; while other portions that could be used for histological study lived for much longer periods.

That the glands were alive and functioning is shown by the progressive changes that occurred as cultivation was continued.¹¹ In many instances the epithelium grew higher and higher, and the colloid became more and more vacuolated. Other glands appeared to be unchanged. Fig. 16 shows a well involuted gland from a patient with Graves' disease, before its cultivation; Fig. 17, the same gland after 14 days' cultivation; and Fig. 18, the same gland after 26 days. The medium with which this gland was perfused contained theelin and extra thyroxin. Another gland, not so well involuted, also taken from a patient with Graves' disease, is shown before its cultivation, in Fig. 19; after 7 days' cultivation, in Fig. 20; and at the end of 27

¹¹ Continuous fall in the pH of the medium necessitated renewal of the perfusate every 2 or 3 days during the first 2 weeks, and at somewhat less frequent intervals later.

days' cultivation, in Fig. 21. In this case the medium was prepared as *originally described*. In the first instance it will be seen that considerable colloid remained at the end of 26 days in the apparatus. In the latter, considerable colloid remained after 7 days of cultivation, but after 27 days hardly any colloid was left.

Fig. 22 shows the same gland as pictured in Figs. 19, 20, and 21, cultivated for 27 days in a medium with a lower concentration of three hormones—eschatin, pitressin, and adrenalin. Considerable colloid is still left in the gland, and the height of the epithelium compares favorably with that after only 7 days in the medium with the higher hormone content. The other illustrations (Figs. 23 to 27) are chosen to show the state of the tissue after quite prolonged periods of cultivation, 35 days in two instances, and 62 days in another.¹² For illustrations showing the condition of the glands at earlier periods in the cultivation, and for a more complete discussion of the results, the reader is referred to the following joint paper with Dr. Foot.

DISCUSSION

All the media used in this work have been found capable of sustaining the life of fibroblasts *in vitro* for 30 days or longer. Therefore, although many of the experiments cited here were of short duration, they illustrate results with media that could presumably support cell life for a much longer time. Some of the difficulties encountered in these short experiments, namely, those with the kidney and suprarenal gland, some of the changes noted with the testicle, and the occasional occurrence of degenerated areas in close juxtaposition to others in which the cells were maintained in excellent condition, appear to be due to accidents, or to conditions attending the cultivation technique, rather than to the fact that artificial media were used. Identical results were observed on cultivating the same glands in 40 per cent serum. Other difficulties, such as the appearance of rather extensive areas of degeneration after prolonged cultivation, may have been due to deficiencies in the nutritive quality of the perfusate. That a medium entirely or almost entirely free from serum proteins and lipoids should function in as efficient a manner as serum is hardly to be expected. Moreover, it is obvious that the medium used here still lacks many substances that are available at one time or another to the organs within the body. Some of these missing substances are undoubtedly essential to the continued life and functioning of the cells.

¹² Although the medium was changed at frequent intervals the possibility that its nutritive action was supplemented by substances coming from the degenerated tissue must be taken into account here.

Nevertheless, some valuable and interesting results have been obtained, which give great promise for what may be accomplished in the future. The human thyroid has been maintained in viable condition for from 2 to 3 weeks with parts of it viable after 5 or 8 weeks, in media entirely devoid of human serum. Some other glands have been maintained for a sufficient length of time to make the pursuit of certain investigations with them possible.

Since the work reported here is the first in this field, it is necessarily incomplete in many respects. The experiments with the thyroid gland have been numerous enough to give a very definite idea of the potentialities of the media. Those with the ovary were too few in number to permit drawing any conclusion concerning the value of the medium for that organ. In all probability the medium, as used, contained too high a concentration of certain hormones. The experiments made with media that were absolutely free from serum show that media that are serumless and yet very nutritious can be used for a short time at least. Again, however, these experiments have been too few in number and continued for too short a time to indicate what the potentialities in this field may be.

There is no doubt that the media which were used here will be altered as work in this field is continued. Some simplifications will probably be made, for in devising these media it was not possible to test in as thorough a manner as would be desirable, the effect of each constituent. Some of the components were tested in tissue culture experiments at many different dilutions. Others were added in groups, at concentrations thought to be within reasonable range as judged by previous experience with other components. Those previously found to be essential to life in growth-promoting media were used in the maintenance media without testing each one individually under the new conditions. But once a medium is found that will maintain life, its simplification becomes a mere matter of routine, though of painstaking work. The more difficult task lies in completing the medium and in providing all the substances that are required for the prolonged life and perfect function of each organ.

It is certain, of course, that media which are much simpler than that used for most of this work will suffice for experiments of short duration. The object in creating this medium was to supply as many as possible of the substances that would be needed for prolonged life; for it is advisable, even in short experiments, to use a medium that nourishes the cells instead of one that merely allows them to survive. Media that will enable one to keep a gland under cultivation for a long time are required, moreover, for all experiments in which one wishes to determine the effect of substances acting continuously at low concentration. Such experiments are prob-

ably of the utmost importance since this may be the way that certain profound changes are produced within the body.

SUMMARY

Experiments have been described in which organs cultivated in the Lindbergh apparatus have been kept alive in artificial media for periods ranging from 3 days to 3 weeks, with life continuing in portions of some glands for from 35 to 62 days. The media used varied in composition from very simple and inexpensive ones in which some serum was used to a rather complex medium containing only that negligible amount of serum that is necessary as a solvent for vitamin A. The experiments performed demonstrate that artificial media can be used for organ cultivation, thus making it possible to study the behavior of human organs; to control the composition of the media at will; and to reduce the cost of experimentation.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Experiment 667. Ovum in a cat ovary cultivated 4 days in medium I. Bouin's picro-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 2. Same experiment as in Fig. 1, showing another portion of the ovary. $\times 206$.

FIG. 3. Experiment 645. Cat thyroid cultivated 6 days in medium I. Zenker-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 4. Experiment 616. Cat thyroid cultivated 6 days in medium II. Zenker-formol fixation. Hematoxylin-eosin stain $\times 412$.

FIG. 5. Experiment 704. Mucosa of uterus of a pregnant cat, cultivated 4 days in medium III. Bouin's picro-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 6. Experiment 796. Mucosa of uterus cultivated 15 days in medium III. Bouin's picro-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 7. Experiment 811. Ovum in a cat ovary cultivated 18 days in medium III with lecithin and cholesterol added. Bouin's picro-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 8. Experiment 714. Epididymis of a cat testicle cultivated 4 days in medium III. Bouin's picro-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 9. Experiment 732. Medulla of the suprarenal gland of a kitten, cultivated 3 days at room temperature in medium III. Zenker-formol fixation. Hematoxylin-eosin stain. $\times 412$.

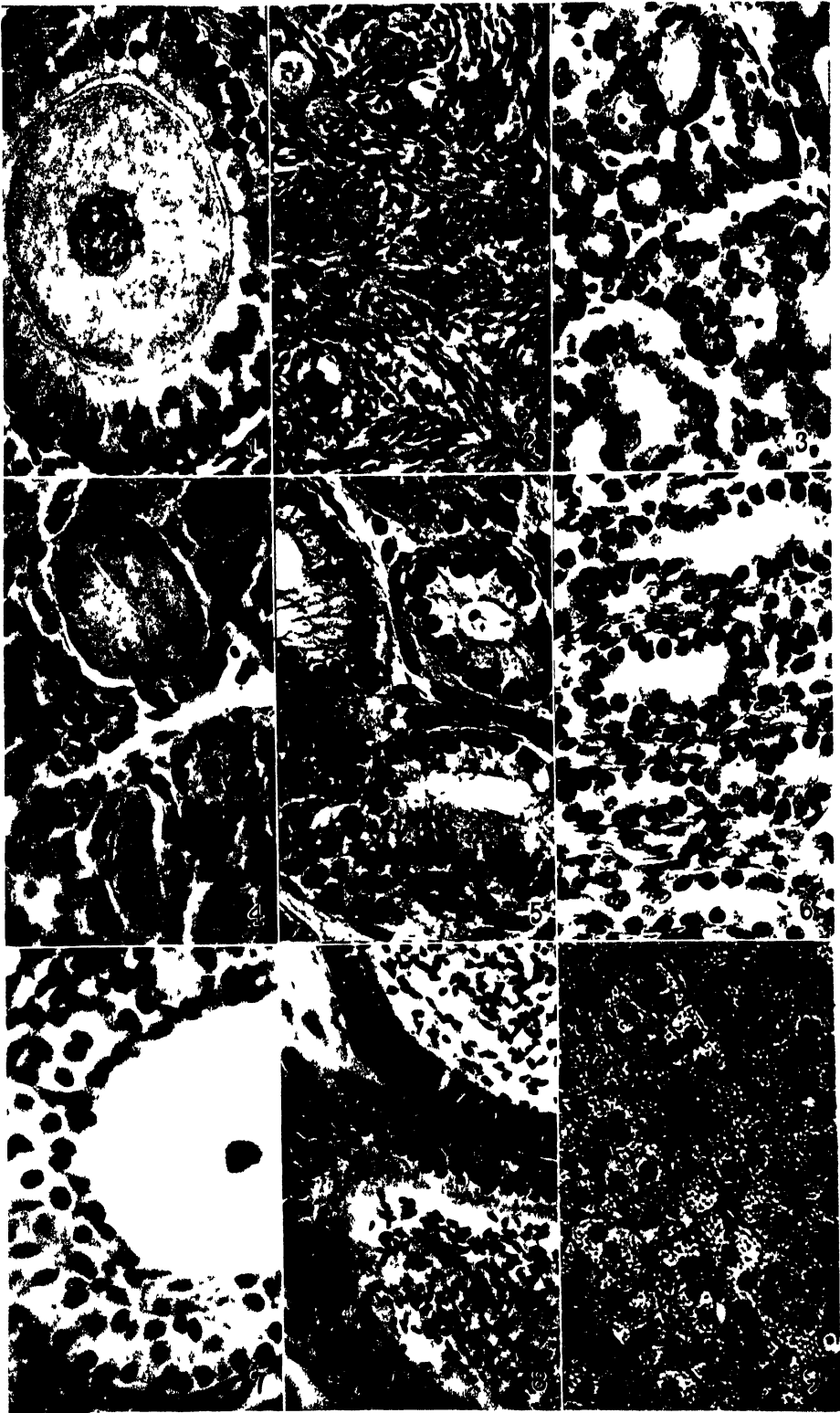


PLATE 2

FIG. 10. Experiment 776. Pelvis of the kidney of a guinea pig embryo, cultivated 5 days in medium III, containing one-half the usual quantity of adrenalin, antuitrin, eschatin, and pitressin. Zenker-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 11. Experiment 793. Cat thyroid cultivated 11 days in medium III, containing one-fifth the usual quantity of eschatin, pitressin, and adrenalin. Cultivation by Ebeling. Zenker-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 12. Experiment 801. Cat parathyroid cultivated 15 days in medium III. Cultivation by Ebeling. Zenker-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 13. Experiment 754. Cat thyroid cultivated 4 days in medium III with the serum, and vitamins A and D omitted. Cultivated by Parker. Zenker-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 14. Experiment 754. Parathyroid gland of a cat, cultivated 4 days in medium III with the serum, vitamins A and D omitted. Same experiment as in Fig. 13. Cultivation by Parker. Zenker-formol fixation. Hematoxylin-eosin stain. $\times 412$.

FIG. 15. Experiment 838. Human thyroid cultivated 10 days in medium III with amino acids substituted for the blood digest and Witte's peptone. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 16. Experiment 856. Control for thyroid shown in Figs. 17 and 18. Uncultivated portion of a thyroid taken from a patient with Graves' disease. Fixed when first procured. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 17. Same gland as shown in Fig. 16 after 14 days' cultivation in medium III, with theelin added and the thyroxin concentration increased. Note the increased height of the epithelium. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 18. Same gland as shown in Figs. 16 and 17 after 26 days' cultivation. Note the still greater height of the epithelium and the greater vacuolation. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

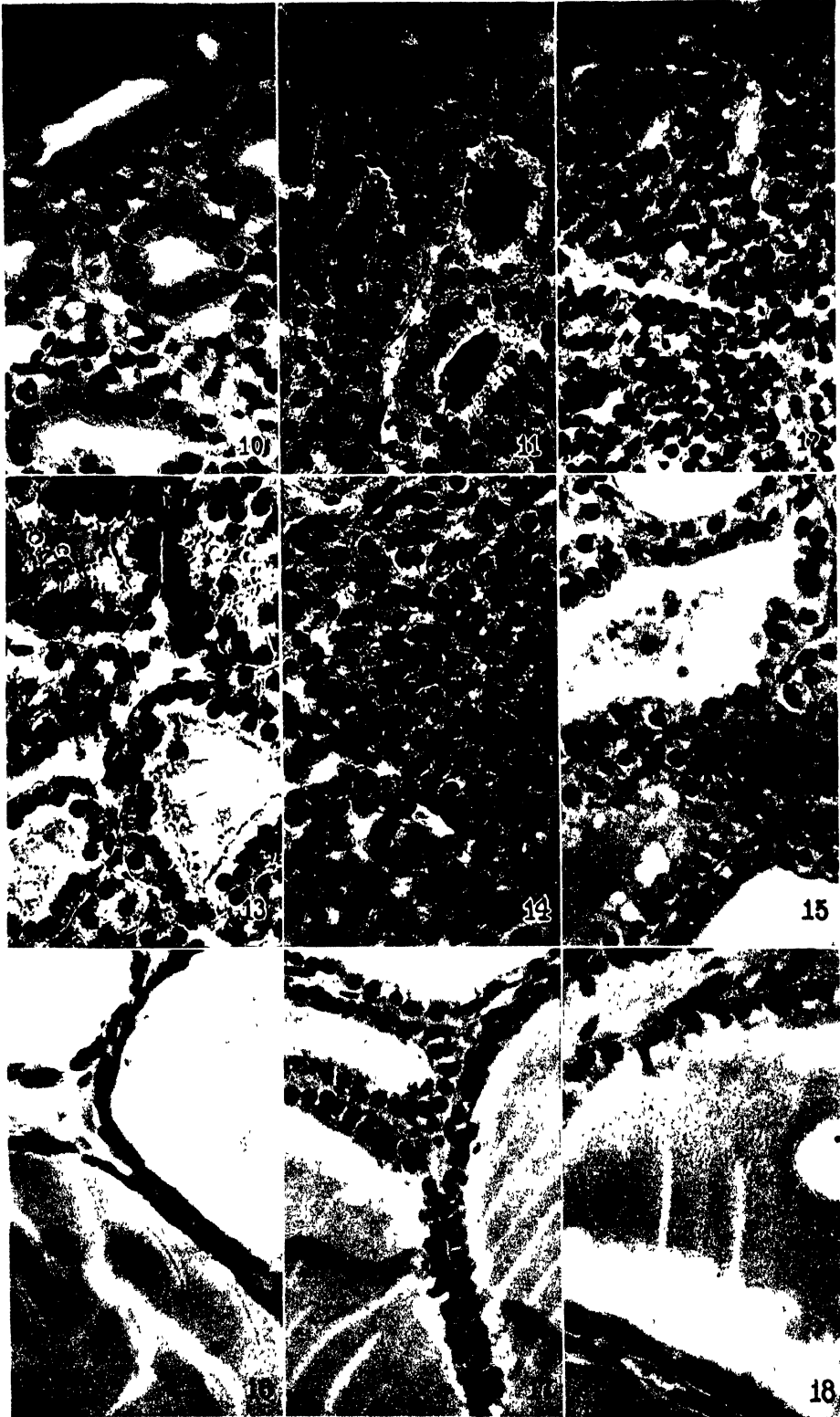


PLATE 3

FIG. 19. Experiment 830. Control for thyroid shown in Figs. 20, 21, and 22. Uncultivated portion of a human thyroid from a patient with Graves' disease. Fixed when first received. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 20. Same gland as shown in Fig. 19 after 7 days' cultivation in medium III. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 21. Same gland as shown in Figs. 19 and 20 after 27 days' cultivation. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 22. Same gland as shown in Figs. 19, 20, and 21 after 27 days' cultivation in medium III with the eschatin, pitressin, and adrenalin reduced to one-fifth the concentration used for the portion of the gland shown in Figs. 20 and 21. This gland was divided and cultivated in two separate pumps. The medium contained no iodine during the first 9 days. Bouin's fixation, Masson modification. Hematoxylin-eosin stain.

FIG. 23. Experiment 835. Control for the thyroid shown in Fig. 24. Uncultivated portion of a gland taken from a patient with Graves' disease and fixed at once. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 24. Same gland as shown in Fig. 23 after 35 days' cultivation in medium III with theelin added. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 25. Experiment 581. Control for the thyroid shown in Fig. 26. Uncultivated adenoma fixed at once. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 26. Same gland as shown in Fig. 25 after 35 days' cultivation in medium III with theelin added. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.

FIG. 27. Experiment 842. Portion of human thyroid still living after 62 days' cultivation in medium III. The medium contained no iodine during the first 14 days. Bouin's fixation, Masson's modification. Hematoxylin-eosin stain. $\times 412$.



THE BEHAVIOR OF ABNORMAL HUMAN THYROID TISSUE CULTIVATED IN THE LINDBERGH APPARATUS

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PLATES 4 TO 6

(Received for publication, March 13, 1939)

The purpose of the experiments discussed in this paper has been to make preliminary observations upon the applicability of the Lindbergh apparatus to the study of human thyroid tissue removed at operation and kept alive in the apparatus for varying periods of time. It will be seen that, by varying the composition of the circulating fluid that is substituted for blood (hereafter called "perfusate" in this paper), valuable clues as to the influence of such substances as iodine and a number of hormones in regulating the metabolism of the gland might be obtained. Once one has found a synthetic medium that can replace human blood and maintain the gland in a condition as nearly similar to that which existed in the body, one may then alter its various constituents, add new ones, and experiment generally on the effect of this or that component. By using such a fluid as a base and by adding or subtracting various constituents, the results we are about to detail were obtained. The work has, of necessity, been rather tentative and not as systematic as that which we propose carrying out in the near future, but it gives definite indications as to the possibilities involved in the use of this method and affords much promise for further experimentation.

Material

The material used consisted of several grams of thyroid tissue removed at operation from the upper pole of one of the lobes of the organ under the usual strict aseptic precautions, care being taken to locate and to preserve a suitable artery that would afford a vascular tree for the explant. Such arteries were usually in the neighborhood of 2 mm. in diameter. The tissue was immediately placed in a sterile fruit jar, the cover clamped on, and the whole well wrapped in a sterile cotton pad a centimeter or so in thickness,

so folded about the jar as to envelope it entirely and to act as a sterile barrier to air infection. Jar and specimen were then transported from the hospital to the neighboring Institute where the specimen was removed, trimmed, treated by immersion in weak sodium hypochlorite solution to preclude surface contamination, and finally set up in the apparatus. Such specimens were kept in this at varying temperatures for several weeks, with a completely artificial synthetic perfusate. The apparatus is described elsewhere (1), as are all the minutiae of manipulation that go with its use. Biopsies about the size of a pea were made from the specimens from time to time under aseptic precautions in the operating room of the Institute. Thus one might examine sections from these at stated intervals and follow the progress of the experiment under the microscope. After each biopsy was taken the tissue specimen was inspected, any bleeding points were tied off, and the tissue returned to the apparatus.

The thyroids used in the experiments varied from pronounced hyperplastic goiters through well involuted cases after Lugolization, to adenomatous and nodular colloid goiters. They were taken as they happened to come to operation and in no particular order. There was one case of struma lymphomatosa among these which, unfortunately, did poorly on the machine and could not be utilized. One carcinoma of thyroid origin was maintained for 8 days at room temperature, but although interesting it is irrelevant to this paper.

Stock Perfusate

The basic perfusate used in the apparatus was devised for this purpose with the aid of the tissue culture technique, and has been described in detail in previous communications (2). It has the following composition, the figures being expressed as percentages in Tyrode's solution as a menstruum.

Blood digest	60 mg. % N	Sodium glycerophosphate	57.3 mg. %
L-tryptophane	5.5 mg. %	Glycerol	0.25 gm. %
Insulin	0.1 unit %	Thymus nucleic acid	20.0 mg. %
Thyroxin	1.0 gamma %	Vitamin A	100 units per 100 cc.
Hemin	4.0 gamma %	Vitamin B ₁	0.1 gamma %
Potassium iodid	Variable	Vitamin B ₂	3.4 gamma %
Cysteine hydrochloride	9.0 mg. %	Ascorbic acid	300 gamma %
Urea	2.4 mg. %	Antuitrin ²	1:500
Glucose	300.0 mg. %	Adrenalin ²	Variable
Casein ¹	0.1 gm. %	Eschatin ²	Variable
Witte's peptone	7.4 mg. % N	Pitressin ²	Variable
Phenol red as indicator	5.0 mg. %		
Glutathione	1.2 mg. %		

¹ Harris purified casein free from vitamins A and B.

² Parke Davis and Company.

In the experiments the amounts of iodine, adrenalin, eschatin and pitressin, nucleic acid, thyroxin, hemin, and insulin were varied. Theelin was added in five experiments and amino acids were substituted for blood digest in one. In one experiment a very simple medium of Tyrode's solution with 2 per cent gelatin and phenol red as indicator was employed.

Microscopic Technique

From each biopsy two fragments were fixed in Bouin's solution containing trichloroacetic instead of acetic acid, one piece being run through at the Institute with hematoxylin-eosin as the stain, the other at the New York Hospital with a modified Masson (Goldner, 3) trichrome technique. Thus the material was available for study at both institutions and any differences in the viability of the tissue, or in the idiosyncracies of technique were equalized.

Interpretation of the Material

We should begin with generalizations applicable to all the experiments, for there were changes in the tissue inherent to the method employed; the sodium hypochlorite produced some surface degeneration of the fragments, blocking of small arterioles by accumulations of coagulated material occurred, and there were other similar artifacts, all of which must be taken into account. In the following descriptions the viable tissue alone is under discussion, the degenerated areas are of no importance in drawing conclusions from the experiments. Neither infection nor degeneration occurred early in any given experiment and the former was uncommon and found to be due to leakage in the gas filtration system, a fault subsequently overcome.

The specimens usually underwent a good deal of shrinkage after being placed on the apparatus, much of which was apparently due to the emptying of stored colloid from the acini, which collapsed and produced a compacted appearance without bringing about much infolding of their epithelium. Rather, the cells seemed to expand as the compressive force of the acinar collapse diminished, so that a flat epithelium became higher, giving the effect of hyperplasia. This elastic effect has been discounted in judging true hyperplasia in these experiments, any mention of hyperplasia will mean just that and not apparent overgrowth. The early emptying of the acini is probably due to the secretion of their contents into the perfusate during the first few days, after which much less is found in the latter by chemical analysis.

Another rather constant feature was the production of very small cells the size of lymphocytes through the degeneration of the acinar epithelium where there was infarction of a "lobule." A small dark nucleus remained, surrounded by a ragged zone of cytoplasm. This has no bearing upon the

production of lymphoid areas in hyperplastic goiter, for a careful check convinces one that these areas have a definite lymphoid reticulum and follicles, while the cells in question merely lie about in groups and lack these features entirely. Although many of the degenerating cells become necrotic, others appear to persist in a fair state of preservation and while this probably has no bearing on "lymphocytic infiltration" in hyperplastic goiter, it might have some in connection with the so called "small cell carcinoma" of the thyroid, often mistaken for lymphosarcoma. A review of numerous sections from a variety of goiters shows this form of degeneration to be relatively common, but as it occurs in small foci it is usually overlooked. Thus it is not peculiar to tissue cultivated in the apparatus, but may occur *intra vitam*.

In studying the experimental material, the nature of the glands under observation was not only taken into account, but the histories of the patients from whom they came were summarized in order to determine if these might have any bearing upon the behavior of the glands subsequent to explantation. As will be seen later, there is no connection noted between the case history and the specimen history. Several specimens were followed over 3 or more weeks and one for 62 days.

One more generality to be noted is the fact that there was a tendency for tissue from hyperplastic glands to undergo involution, becoming less hyperplastic and more normal in appearance or to remain unchanged in the apparatus, unless certain introduced substances were present in the perfusate. This would indicate that the stimulus for hyperplasia is extrinsic, rather than intrinsic in the gland itself.

EXPERIMENTS

Simplest Medium

Tissue from a nodular colloid goiter was placed on circulation with a fluid composed of 2 per cent gelatin in Tyrode's solution and 5 mg. per cent phenol red as an indicator. It was maintained on this lean medium for 13 days, biopsies being taken on the 5th and 13th. The first showed changes of staining affinity in the colloid from red to green in the Masson-stained sections, which is interpreted as an increased fluidity of the colloid which, when it is more inspissated, should stain red or orange. The colloid was also coagulated and fragmented into clumps and irregular masses, such as are seen in degenerating goiters. The epithelium became somewhat vacuolated. At 13 days it was flattened and opaque, with an increased refraction index. There was little of the focal degeneration seen in other specimens, the process appearing to represent a slow starvation of the tissue

on a medium too lean in nutriment. This experiment might serve as a base line for those that follow; the tissue was remarkably well preserved and free from infection, it merely had a shrunken and somewhat coagulated or desiccated appearance.

Effects of Iodin on Explanted Tissue

100 gamma per cent was supplied in the form of potassium iodid in twelve experiments, there was none in five, one began with none for 9 days, after which 100 gamma per cent was added and two ran with none for 14 days, after which the same amount was added. The basic medium was present in all cases, but some of its constituents other than iodine, notably the hormones, varied in their concentration.

Hyperplastic Goiter (Exophthalmic Goiter).—Of thirteen glands that were hyperplastic when removed at operation, eight either continued to be so or grew more hyperplastic on a perfusate containing 100 gamma per cent of iodine; one showed no change until the iodine was added on the 9th day, whereupon it became hyperplastic; three glands were unchanged in the presence of 100 gamma per cent and one showed hyperplasia in its total absence.

Adenomatous and Nodular Colloid Goiters.—In the presence of 100 gamma per cent of iodine one showed no change, while in its absence two showed no change and two slight hyperplasia.

In evaluating these results it is necessary to consider other factors, when it will be seen that iodine apparently played no consistent part in favoring or inhibiting hyperplasia in the tissue in the apparatus. Nine out of thirteen hyperplastic glands showed continuing hyperplasia in its presence, which might at first seem significant even though surprisingly contradictory to clinical experience. It may be that iodine does not work upon the epithelium through immediate contact and action when administered in the form of Lugol's solution to the patient, but rather by reason of some intermediate action on another endocrine organ. Or it may be that its action in these experiments was entirely overbalanced by the stimulating action of some other component of the medium. If the iodine had any influence on the glandular epithelium, in our experiments, it was to favor rather than to inhibit hypertrophy.

Effect of Adrenalin on the Tissue

The amount of adrenalin in the perfusate varied from none to 100 gamma per cent. In two experiments there was none, in fourteen there was 20 gamma per cent, and in six, 100 gamma per cent.

Hyperplastic Goiters.—With no adrenalin, one gland showed slight hyperplasia, another marked metaplasia and slight hyperplasia, which will be discussed later. With 20 gamma per cent, six showed continuing hyperplasia, two a peculiar vacuolization of their cytoplasm, and two little change. With 100 gamma per cent three showed continuing hyperplasia and one but little change.

Other Goiters.—Five showed no significant change with 20 gamma per cent and with 100 gamma per cent one showed slight hyperplasia and one none at all.

These figures are also without any particular significance when one takes into account other factors involved. The adrenalin content of the perfusate appears to be of relatively little importance.

Effect of Eschatin and Pitressin

Eschatin is the Parke Davis preparation of cortical suprarenal hormone, pitressin a posterior hypophyseal lobe hormone that had been used in other experiments and was left in combination with the eschatin in these. They were used in dilutions of 1:5000 and 1:1000.

Hyperplastic Goiters.—Of those subjected to a 1:5000 concentration of these hormones, four showed no marked change in the epithelium and one was unchanged until the perfusate, which had contained no iodine, had 100 gamma per cent added, whereupon it became hyperplastic. In the lesser dilution of 1:1000, ten hyperplastic goiters remained so or became more hyperplastic (some of them having already undergone more or less complete involution in the patient before operation).

Other Goiters.—Of these three showed no change with a 1:5000 concentration, while one showed slight hyperplasia. One remained unchanged in the presence of a 1:1000 dilution for a few days and then slowly became hyperplastic.

Here, then, we have results that are striking and consistent. There appears to be a definitely stimulating action on the part of one or the other of these hormones. This is illustrated in Figs. 1 to 8.

Effects of Nucleic Acid

This was used in concentrations of 0, 10, and 20 mg. per cent. There is no need to detail the results as they were inconsistent and quite unilluminating, apparently this element was largely without effect.

Influence of Insulin

Insulin was regularly present in a concentration of 0.1 unit per cent in all the experiments, in one experiment it was increased to 5 units per cent.

The most significant fact to be gleaned from this one instance was that the material used failed to show marked hyperplasia although the eschatin-pitressin concentration was high and theelin was present. This was the only primarily hyperplastic goiter that failed to show definite and continued hyperplasia in the presence of high concentrations of these hormones, which might indicate an inhibitory action on the part of the insulin.

Effects of Theelin

In five experiments tissue from two hyperplastic, one involuting hyperplastic goiter, and one adenoma was placed on a perfusate containing 0.4 gamma per cent of theelin, the thyroxin content being increased from 1.0 gamma to 1.55 gamma per cent. In one of these the tissue showed progressive and marked hyperplasia, the most marked overgrowth to be noted in the series. It had the appearance of an untreated hyperplastic goiter. This might point to the presence of theelin were it not for the contradictory evidence of the other four experiments which showed no comparable hyperplasia. In all five of these experiments the eschatin-pitressin concentration was also high.

Effects of Thyroxin

In one of the five experiments in which the amount of this substance was increased there appeared vacuolization of the epithelium and hyperchromasia of the nuclei, with marked disparity in the size of the cells, some of which were nearly twice as large as their fellows. The nuclei tended to bulge out into the acinar lumina beyond the level of the cytoplasm. Later, however, the latter increased in amount and showed vacuolization. At the same time it became somewhat refractile, giving the cells the hyalin appearance characteristic of the epithelium in struma lymphomatosa, or in that of the involuting thyroids of cretinism or myxedema. The insulin concentration was also high in this experiment (5 units per cent), which should be taken into account. See Figs. 9 and 10 for illustrations of this experiment.

Effect of the "Human Element"

In those experiments employing hyperplastic goiters, the histories of the cases were reviewed in order to determine whether there was any correlation between the past history and subsequent developments in the apparatus. The cases were divided into severe, moderate, and mild groups on the basis of the symptoms and the basal metabolic rates.

Severe Cases.—There were three in this group. The first had a very turbulent course and died as the result of a postoperative "thyroid storm;" her B.M.R. averaged 46 for seven readings, between 36 and 65. In spite

of this, her tissue showed no hyperplasia in the apparatus where it was maintained for 9 days on a fluid containing low eschatin-pitressin concentration. A control section from the gland at operation showed generally good involution under Lugolization, with a few areas of persisting hyperplasia. The second case had moderate symptoms, but average 46 B.M.R. for four readings. The tissue in the apparatus showed hyperplasia throughout 15 days, after which it degenerated. The eschatin-pitressin concentration was high. Control sections showed moderate hyperplasia. The third patient had moderate symptoms and an average B.M.R. of 54 on two readings. The tissue involuted and showed no hyperplasia while in the apparatus for 9 days, with a low eschatin-pitressin concentration. Control sections showed definite hyperplasia at the time of operation.

Moderate Cases.—The first had a B.M.R. of 27 on five readings with moderate symptoms. The tissue was hyperplastic in the machine where it was followed for 13 days, after which it degenerated. The eschatin-pitressin concentration had been high. The control sections showed good involution. The second case had an average B.M.R. of 37 for five readings and moderate symptoms. The tissue showed the most marked hyperplasia of the series and was followed in the apparatus for 35 days, when there was still viable tissue present. Here, the eschatin-pitressin concentration had been high and theelin was present in the perfusate. Control sections showed irregular involution. In the third case the B.M.R. averaged 43 for four readings and symptoms were mild. Death followed postoperative atelectasis. The tissue was followed on two machines for 16 and 11 days respectively; in one case it showed hyperplasia in the presence of a high eschatin-pitressin concentration, while in the other, with a low concentration of these hormones, it showed little change. Control sections showed moderate hyperplasia. The fourth case had an average B.M.R. of 32 on three readings and moderate symptoms. The tissue became metaplastic rather than hyperplastic in appearance, resembling a malignant neoplasm somewhat. The eschatin-pitressin concentration was high and two fragments were followed on two machines for 29 and 62 days respectively, both showing the unusual change. Control sections showed good involution at the time of operation. The fifth case was of considerable interest as the patient was emotionally unstable and showed wide fluctuations in her B.M.R. (30-70), the average being 47 for ten readings. The tissue became definitely hyperplastic on the apparatus and this continued and increased during the 27 days it remained there, five biopsies being taken during this period. The eschatin-pitressin concentration had been high, control sections showed a gland that was essentially normal, with mature,

unscalloped colloid and low epithelium. The sixth case was a Chinese patient with an average B.M.R. of 40 on seven readings. His tissue, maintained on two machines, showed hyperplasia that came on slowly after the 9th and 12th days respectively. The tissue remained in the apparatus for 27 days in both instances. The eschatin-pitressin concentration was high in one, low in the other; there was no iodine present in that with the low hormone concentration until the 9th day, when 100 gamma per cent was added. In that with the high hormone concentration iodine was present in this concentration from the start. Control sections showed moderate hyperplasia persisting after Lugolization and the patient had diabetes as a complication.

Mild Cases.—There were two of these, the first had an average B.M.R. of 23 on three readings with mild symptoms. The tissue was divided between two machines, one having the usual 0.1 unit per cent of insulin, the other 5 units per cent, with the eschatin-pitressin concentration high and theelin present. The thyroxine and hemin were also increased in both instances. They were followed for 5 and 36 days respectively. The tissue showed the changes described under "thyroxine." Control sections showed hyperplastic goiter without much involution. The second mild case had an average B.M.R. of 17 for eight readings and mild symptoms. Tissue in the apparatus showed slight hyperplasia that remained practically stationary during the 47 days that it was followed. Eschatin-pitressin concentration was low. The control sections at time of operation showed well involuted hyperplastic goiter.

These results are of interest as they apparently demonstrate that there is no definite correlation between past history, the state of the tissue when removed from the patient to the apparatus, and the subsequent behavior of this tissue in the apparatus. In every instance the hyperplasia that was marked and progressive was seen coincidentally with high concentration of eschatin-pitressin. Reference to these brief protocols will show that those glands that showed marked hyperplasia on the machine, might show little in the control sections and that the patient might come from the moderate group. The third severe case, for example, showed high B.M.R. readings and an uninvoluted, typical hyperplastic goiter at operation. Nevertheless, tissue from this gland involuted in the apparatus in the presence of a low eschatin-pitressin concentration.

Metaplastic Changes

One of the experiments showed very interesting metaplasia that is unaccountable but should be mentioned. This was an exophthalmic goiter

from one of the moderate cases (case 4, moderate), control sections from which showed a good deal of variability in their pictures, but nothing striking. After a few days in the machine hyperplasia became marked and metaplasia appeared, to increase steadily until the sections looked enough like carcinoma to deceive a competent thyroid surgeon who examined them into thinking that they were cancer. After 34 days the metaplasia was very marked, but during the last few weeks in the apparatus the gland became more normal looking and was merely hyperplastic at the end of 2 months. Iodin had been added to the perfusate at the 14th day, prior to which none had been present. There were ample controls for the high hormone-low iodine combination, none of which reacted similarly. There is nothing in the history of the case that might explain it and the patient made an uneventful recovery from the operation. In all probability we are dealing with some sort of degenerative change leading to metaplasia, a phenomenon sometimes seen in "burnt out" cases of exophthalmic goiter with exhaustion atrophy (Figs. 11 and 12).

Effects on Thyroid Colloid

In all the material the appearance of the colloid in the acini was carefully noted and checked against the various factors that might be responsible for the changes noted. The only artifacts seen were in connection with extensive degeneration, when colloid might become granular and disappear. It was also found that necrosis of the epithelium frequently left the colloid unaffected. It has been found that normal colloid has an affinity for the red and orange elements of the Masson-Goldner stain and presents smooth, rounded contours. Not infrequently portions of it will take the green stain. In hyperplastic goiters, however, most or all of the colloid stains light green and exhibits vacuolated or scalloped margins. It was found that in nineteen experiments there was a definite relationship between the presence of hyperplasia and green-staining colloid on the one hand and high concentration of eschatin-pitressin on the other. Conversely, in the presence of low concentration of these hormones the red-staining colloid tended to persist for several to many days. Scalloping followed the same lines. It was interesting that the eschatin-pitressin concentration could be accurately judged (high or low) by the appearance of the colloid alone.

DISCUSSION

It should be emphasized at the outset that the purpose of these experiments was primarily an attempt to ascertain whether the methods employed might offer promise for further investigation of human tissue problems,

rather than to set up a series of tests calculated to answer any questions as to the influence of hormones or other substances on the surviving thyroid. This promise seems to have been fulfilled, for human tissue has been transplanted to the apparatus and has kept in excellent condition for sufficient time periods for one to carry out a variety of experiments of the sort described.

So far as results obtained from chemical alterations in the perfusate are concerned, it must be admitted that it would be presumptuous to try to draw any definite conclusions when so many variables are present. One of the substances that was present in this perfusate, the combined eschatin-pitressin content, seems to have yielded striking results, however, that should be given due consideration. Another point that stands out from the rather confused mass of data is the independence of the behavior of the thyroid after transplantation of its past behavior in the body, once subjected to the new surroundings of the apparatus the tissue seems to react purely to the type of perfusate employed and not to carry over any inherent tendency to undergo hyperplasia. On the contrary, hyperplastic glands have seemed to quiet down in the apparatus once they were removed from the body, provided that the perfusate did not contain any stimulating substance.

This behavior is of importance in view of the uncertainty that exists as to the cause of hyperplastic goiter. A perusal of Rienhoff's (4) article in Lewis' "Practice of surgery," or of Boyd's "Surgical pathology" (5) will convince the reader that there is still much difference of opinion concerning this point.

The effect of a high concentration of eschatin and pitressin (1:1000) in the perfusate stands out in a clear cut fashion throughout the experiments. It produced definite hyperplasia irrespective of the condition of the tissue when transplanted to the apparatus, it had its effects upon the absorption of the colloid from the acini, which took on the green-staining characteristics regularly noted in hyperplastic goiter.

Results obtained by varying other constituents of the perfusate were not constant and are mentioned in the body of the paper for the sake of completeness, rather than from any desire to show that they prove anything. It is interesting that a high iodine content, if it did anything, seemed to favor hyperplasia, rather than to inhibit it; this is the opposite of what one might expect from well known experiences with iodine therapy in exophthalmic goiter. So far as insulin is concerned, it is again interesting that a gland maintained on a high eschatin and pitressin concentration, with theelin also present, failed to develop the usual hyperplasia when the in-

View of the Experiments

Experiment No.	Iodin	Adrenalin	Dilution epinephrin-pitressin	Nucleic acid	Changes in medium	Clinical diagnosis	Pathological diagnosis	Results in the apparatus	Time followed in apparatus
	gamma per cent	gamma per cent		mg. per cent					days
808	100	20	1:5000	20	None	Graves' disease	Involuted hyperplasia	Slight hyperplasia	47
809	100	100	1:1000	20	"	" "	Hyperplastic goiter	Hyperplasia	8 degeneration
810	100	100	1:5000	20	Embryonic chick extract added	" "	Moderate hyperplasia	Unchanged, involution	9
812	100	20	1:5000	20	Heated serum added on 4th day	Nodular colloid	Nodular colloid	" "	20
Run at room temperature, 25°C.									
813	0	100	1:1000	20	15th day 10 gm. % iodine	Graves' disease	Moderate hyperplasia	Hyperplasia	30
816	0	100	1:1000	20	None	Nodular colloid	Nodular colloid	Slight hyperplasia	17 degeneration
823	0	20	1:5000	10	"	Graves' disease	Hyperplastic goiter	Unchanged, involution	9 infection
824	0	20	1:5000	10	"	Adenoma	Degenerated adenoma	Hyperplasia	3 infection
829	0	20	1:5000	10	9th day increased iodine and nucleic acid	Graves' disease	Moderate hyperplasia	Unchanged to 9th then hyperplasia	36
830	100	20	1:1000	20	None	Same	Same gland	Hyperplasia after 12 days	27
835	100	20	1:1000	20	Theelin and increased thyroxine	Graves' disease	Hyperplastic goiter	Marked hyperplasia	35
Run 1 wk. at 37.5°, then at 25°									
836	0	20	1:5000	10	None	Adenoma	Adenoma	Unchanged	11 infection
838	100	20	1:1000	20	Amino acids replace blood digest	Graves' disease	Moderate hyperplasia	Hyperplasia after 7 days	16 degeneration
839	100	20	1:5000	20	None	" "	" "	Unchanged	11 degeneration
841	0	100	1:1000	10	14th day 100 gm. % iodine	" "	Involuted hyperplasia	Metaplasia, hyperplasia	29
842	0	0	1:1000	10	" "	Same	Same gland	Metaplasia and hyperplasia	62
851	0	0	1:1000	0	Theelin and increased thyroxine and hemin	Adenoma	Adenoma	Hyperplasia	35
856	100	20	1:1000	0	" "	Graves' disease	Well involuted	"	26 infection
861	100	20	1:1000	0	" "	" "	Hyperplastic goiter	Hyperplasia and vacuolated cytoplasm	3
862	100	20	1:1000	0	5 units % insulin Theelin and increased thyroxine and hemin	Same	Same gland	" "	36
863	0	0	0	0	Tyrod's solution 2% gelatin	Toxic nodular	Moderate hyperplasia	Involuted, slow atrophy	13

sulin content was increased to 5 units per cent. It may be asked why the thyreotropic hormone of the pituitary was slighted in this series of experiments. It had already been used in another series of experiments (Okkels, 6, 7) and was therefore omitted in ours. The thyroids of cats and rabbits were found to show some hyperplasia following its use, but this was somewhat less than that which might be noted in living animals.

A tabular view of the experiments is presented, in order to summarize the work briefly and to afford an easy way of comparing the experiments with one another.

CONCLUSIONS

1. Human thyroid tissue may be kept alive and in good condition in the Lindbergh apparatus for at least 3 weeks, in usable condition from 1 to 2 months, provided that the vascular tree remain patent and functioning.

2. Experiments with altered amounts of iodine, adrenalin, hormones, and other substances show constant results only in connection with eschatin and pitressin which, in concentrations of 1:1000 in the perfusate almost invariably cause hyperplasia of the explanted tissue.

3. The original state of the tissue and the character of the symptom complex in the patient from which it was taken appeared to play no part in the subsequent behavior of the tissue explants.

4. In one experiment a most remarkable metaplasia was observed in the explanted thyroid tissue during the 6 to 8 weeks that it remained in two apparatuses. The cause of this is not evident.

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EXPLANATION OF PLATES

PLATE 4

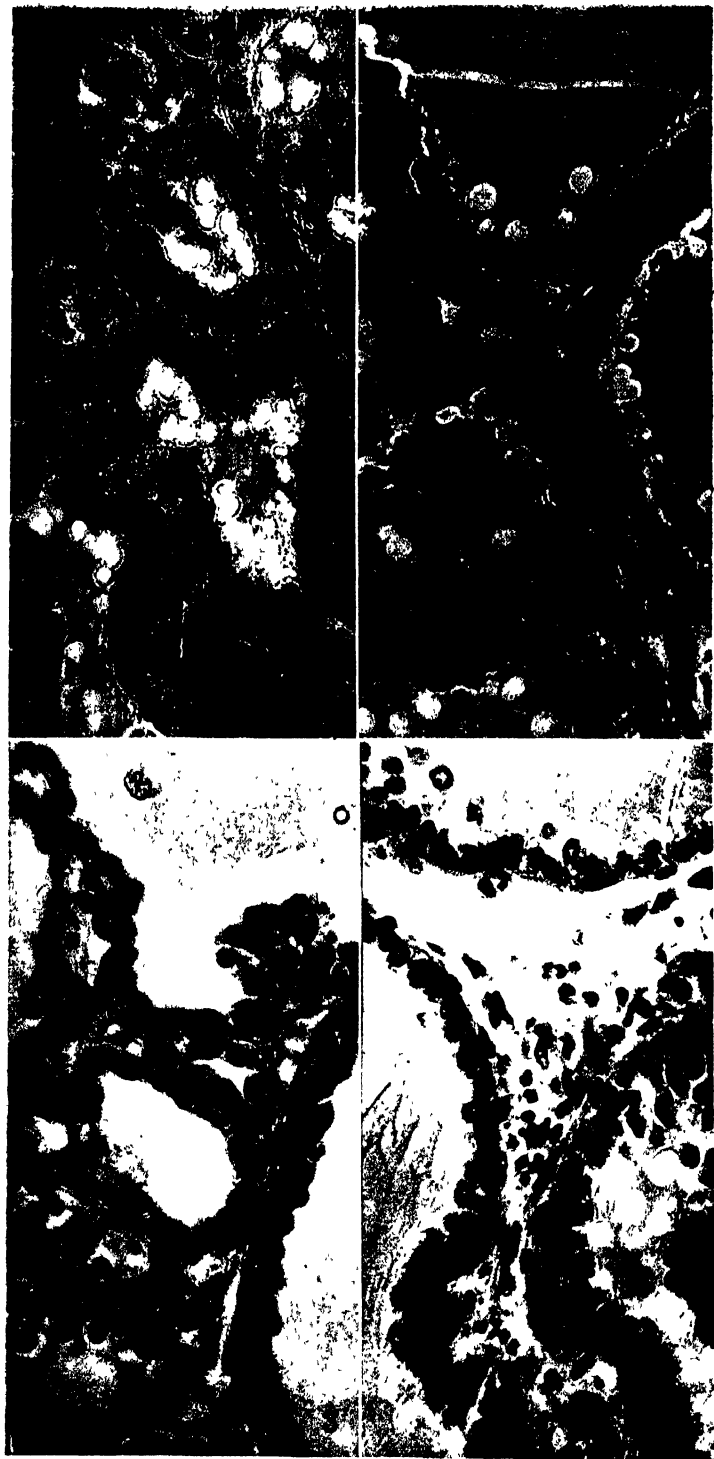
FIG. 1. Control section from thyroid before cultivation in the apparatus. $\times 420$.

FIG. 2. Section from biopsy on 3rd day of cultivation with a perfusate low in eschatin-pitressin (1:5000). $\times 420$.

FIG. 3. Same material after 7 days' cultivation on the same perfusate, showing notable involution of the epithelium which is lower and definitely cuboidal. Compare with control. $\times 420$.

FIG. 4. Section from biopsy on 9th day of cultivation. There is no hyperplasia of note. $\times 420$.

All photomicrographs were made at The Rockefeller Institute, with the exception of Fig. 12, which was taken at the Department of Photography of the Cornell University Medical College.



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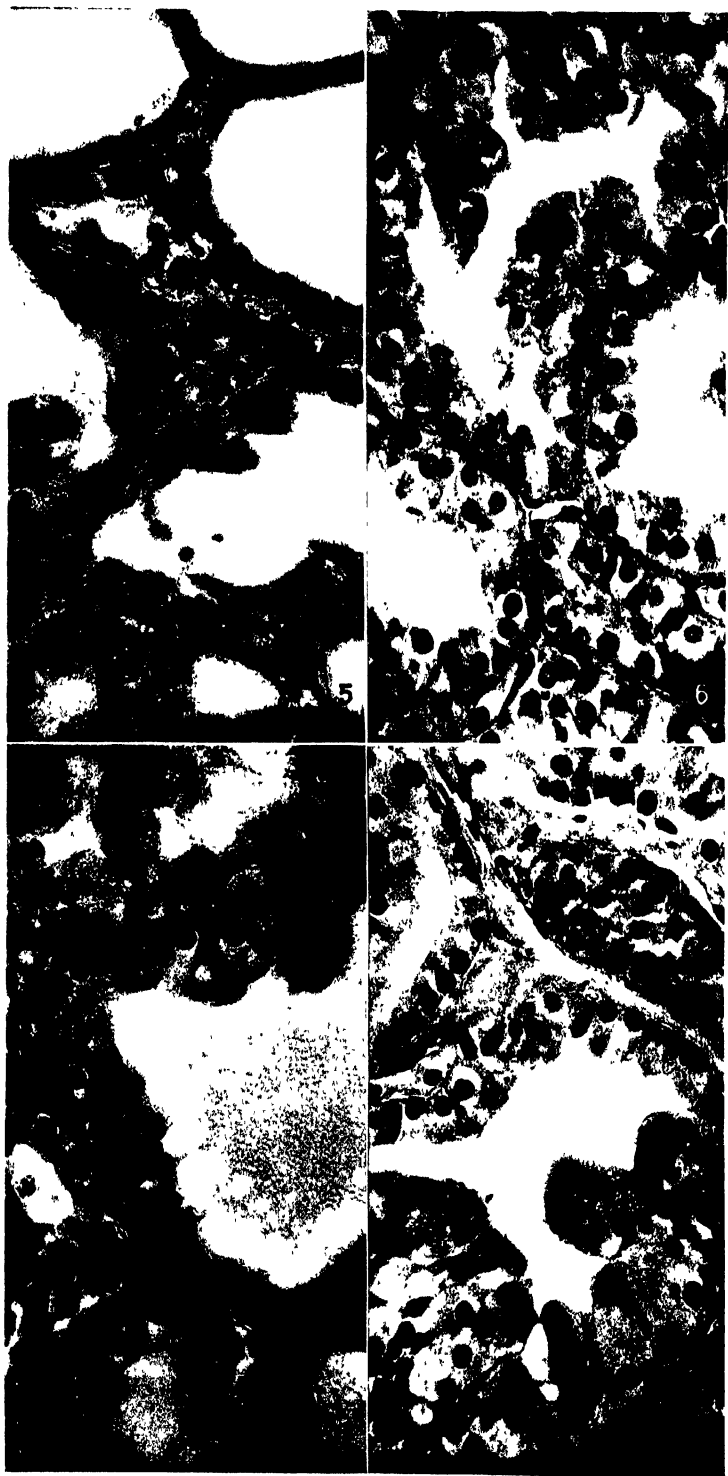
PLATE 5

FIG. 5. Control section from thyroid before cultivation in the apparatus. In this instance the gland shows fairly good involution following Lugolization of patient. Compare with the control of the preceding experiment, where involution was poor and the gland hyperplastic. $\times 420$.

FIG. 6. Biopsy on 5th day after cultivation in the apparatus on a perfusate with a high concentration of eschatin-pitressin. Note that there is already marked hypertrophy of the epithelial cells and almost total disappearance of the colloid. $\times 420$.

FIG. 7. Biopsy on 9th day of cultivation. The hyperplasia continues. $\times 420$.

FIG. 8. Biopsy on 12th day of cultivation. Hypertrophy and hyperplasia at their height. Compare with the control and note the marked change after 12 days on the high concentration of hormones. $\times 420$.



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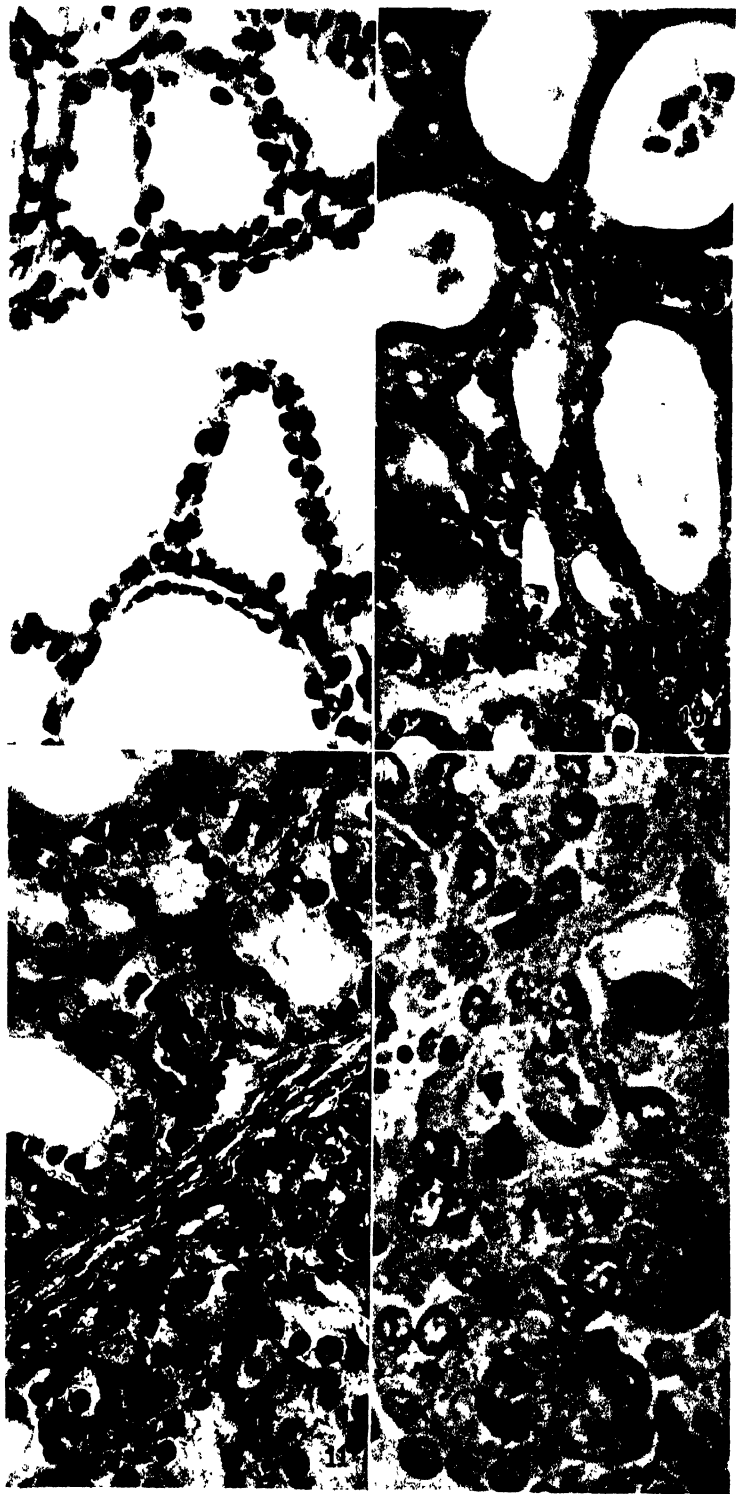
PLATE 6

FIG. 9. Biopsy from gland cultivated for 3 days on a medium with 5 units per cent of insulin and increased thyroxin. Note the disparity in the size of the cells, with vacuolization of the cytoplasm and the very low, endothelioid type of the epithelium. $\times 420$.

FIG. 10. Biopsy on 36th day from the same gland. Here one notes a peculiar density of the cytoplasm, which has become refractile. Although many nuclei are somewhat pycnotic, there are others which show the cells to be in good condition. $\times 420$.

FIG. 11. Control section from thyroid at time of operation. It is irregularly hyperplastic, but it shows no metaplasia. $\times 420$.

FIG. 12. Biopsy from the same gland on the 5th day of cultivation on a perfusate with a high concentration of eschatin and pitressin, 100 gamma per cent of adrenalin, no iodine, and 10 mg. per cent of nucleic acid. The reason for the marked metaplasia that is evident in this figure is quite unknown. After adding 100 gamma per cent of iodine on the 14th day this metaplasia continued for a week or so and then gradually gave way to a return to a more normal looking architecture. $\times 530$.



(Foot *et al.*: Abnormal human thyroid tissue in Lindbergh apparatus)

THE ENHANCING EFFECT OF AZOPROTEINS ON THE LESIONS PRODUCED BY VACCINE VIRUS, THE SHOPE FIBROMA VIRUS, AND THE AGENT TRANSMITTING CHICKEN TUMOR I

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PLATES 38 AND 39

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It has been noted that the lesions produced in the skin of rabbits by the injection of various infectious agents are markedly enhanced by the addition of testicular extract (1). The phenomenon is now known to depend on a factor which causes intradermally injected material to spread extensively through the dermis (2). A similar factor capable of increasing tissue permeability can be detected in extracts derived from a variety of other animal tissues and from certain microorganisms (3). Azoproteins have been shown to produce a like effect, as far as increasing skin permeability is concerned (4), but whether or not the mechanism involved in the production of the spread is the same for the two classes of substances, has not been determined (5). For further comparison the effect of azoprotein solutions has been tested on the lesion produced by vaccine virus, the Shope fibroma virus, and the agent transmitting Chicken Tumor I.

Material and Method

Azoproteins.—The azoproteins were prepared by following closely the procedure given in a previous paper (4). In the present work the azo compounds used were produced by the coupling of *p*-diazobenzenesulfonic acid with horse or rabbit serum proteins, or with egg albumin. Gelatin, which is practically lacking in coupling power, was also used in a few tests. Neutralized solutions of *p*-diazobenzenesulfonic acid, serum, and egg albumin solutions at the proper concentrations, were tested separately as controls.

Test for Spreading Power.—As a rule, the spreading power of the azoprotein solutions tested for enhancing properties was also determined, usually in the same animal, and in a corresponding area of the skin. For these tests, 0.5 cc.

of the azoprotein solution was mixed in a syringe with 0.25 cc. of India ink previously diluted with 2 volumes of water, and the mixture was injected intracutaneously. The results were recorded by measuring the area of spread 18 to 24 hours after the injection. Since azoproteins are colored products, their spread through the dermis could be estimated also directly, through discoloration of the skin in the area of diffusion, without need for the addition of India ink.

Vaccine Virus.—The vaccine virus used in the present tests was a subculture of an *in vitro* strain (B. P. H. culture 662, Oct. 16, 1934), kindly supplied by Dr. T. M. Rivers. The infective material had been stored in 50 per cent glycerin and, before use, was diluted with Ringer's solution.

Shope Papilloma Virus.—The original fibroma virus was kindly supplied by Dr. R. Shope (6). The material used in these experiments was obtained by the subpassage of the virus in the skin of rabbits. The freshly removed fibroma tissue was ground with sand and extracted with distilled water in the proportion of 5 to 10 cc. water per each gram of tissue. The extract was then centrifuged and filtered through sterile gauze.

Chicken Tumor Agent.—Fresh Chicken Tumor I tissue was ground with sand and extracted with sterile distilled water in the proportion of 10 to 12 cc. water per gram of tissue. After centrifugation at 3000 R.P.M. for 10 minutes, the extract was filtered through sterile gauze. In some instances the inhibiting factor was removed from the extract by adsorption with aluminum hydroxide (7).

Tests for Enhancing Power of Azoprotein Solutions.—The enhancing property was tested by mixing an equal volume of the azoprotein solution with dilutions of the virus suspensions. Of these mixtures 1 cc. was injected subcutaneously in rabbits. Virus preparations diluted with an equal volume of saline solution or with untreated protein solutions served as controls.

The chicken tumor extract was mixed in the syringe with an equal volume of the test solutions, and 0.6 to 0.8 cc. of the mixture was injected intracutaneously into adult Plymouth Rock hens. Since tissue diffusion caused both by testicular extract and azoproteins is influenced by gravity, in order to allow sufficient space for the material to spread, the injections were made just under the wing or in the skin covering the upper part of the leg.

Effect of Azoproteins on Vaccine Virus Lesions

The results of tests with azoproteins and varying dilutions of vaccine virus compared with the areas of spread of the azoprotein with India ink are shown in Table I.

It will be noted that azoprotein increases to a considerable degree the size of the lesion produced by vaccine virus. The results indicate that the extent of the lesion depends not only on the spreading power of the azoprotein, but also on the potency of the infective material. As a rule, the more concentrated virus preparation with azoprotein gave, 6 to 7 days after inoculation, wide, solid lesions surrounded

by a zone of edema. This condition is illustrated in Fig. 1, where the size of the enhanced lesions was 67.6 sq. cm. compared with 10.5

TABLE I
Effect of Azoproteins on the Size of the Lesions Produced by Vaccine Virus in the Rabbit Skin

Experiment No.	Azoproteins prepared from sulfanilic acid and	Dilution of vaccine virus	Area of spread of India ink indicator and		Area of 7 day old lesions produced by vaccine virus and		Character of virus lesions on azoprotein side
			Saline	Azoprotein solution	Saline	Azoprotein solution	
			sq. cm.	sq. cm.	sq. cm.	sq. cm.	
1	Egg albumin (3% sol)	1/10	5.5	87.0	10.5	67.6	Solid mass surrounded by scattered nodules and zone of edema
2	" " " "	1/10	4.2	60.7	14.0	34.0	Solid mass
	" " " "	1/100			7.5	27.6	Only scattered nodules
	" " " "	1/1000			0	16.6	" " "
3	Gelatin (3% sol)	1/10	6.8	41.8	12.4	0	No lesions (virus inactivated)
4	Horse serum	1/10	1.8	90.3	6.7	82.8	Solid mass surrounded by scattered nodules and edema
5	" "	1/10	8.0	76.7	4.6	44.6	Solid mass
	" "	1/100			2.2	25.5	Solid mass plus scattered nodules
	" "	1/1000			0	17.0	Scattered nodules
6	" "	1/10	6.2	58.0	20.8	56.0	Solid mass
	" "	1/100			10.2	46.8	Solid mass plus scattered nodules
	" "	1/1000			1.8	48.1	Only scattered nodules
7	Rabbit serum	1/10	3.4	36.8	6.7	39.1	Solid mass
	" "	1/100			3.4	27.6	Solid mass plus scattered nodules
	" "	1/1000			2.1	20.8	" "

sq. cm. for the saline control. The azoprotein used with the vaccine virus was tested for spreading power in another rabbit and gave, 24

hours after the injection, an area of spread of 87 sq. cm. against 5.5 sq. cm. for the saline control (Fig. 2).

The injection of dilute virus with azoprotein gave rise to scattered nodules, with or without a solid lesion at the site of injection.

In two instances, vaccine lesions developed from the azoprotein-virus mixtures while the virus-saline inoculum remained negative. This evidence of real enhancement of the virus, as the result of the spread, was also observed with testicular extract by Duran-Rey-

TABLE II

Effect of Azoproteins on the Size of Fibroma (Shope) in the Rabbit Skin

Ex- peri- ment No.	Azoprotein prepared from sulfanilic acid and	Area of spread of India ink indicator plus		Area of 7 day old lesions produced by fibroma virus plus		Character of lesions on azoprotein side
		Saline or normal serum	Azo- protein solution	Saline or normal serum	Azo- protein solution	
		sq.cm.	sq.cm.	sq.cm.	sq.cm.	
1	Horse serum	7.5	77.0	5.2	71.4	Solid mass, surrounded by a few scattered nodules
2	" "	5.7	43.1	4.0	35.7	Solid tumor
3	" "	6.1	62.4	5.6	54.3	Solid tumor, surrounded by isolated nodules

nals (8). It is possible that the presence of the tissue extract or the azoprotein solution stabilizes the virus at the highest dilutions, and protects it from inactivation. Such an action would explain the apparent enhancement of the virus itself.

Azoproteins prepared from serum and from egg albumin were identical in their effect. A gelatin solution, submitted to the same procedure of coupling exhibited some spreading power, but the vaccine virus when added to the latter, was completely inactivated. The virus was also destroyed by the neutralized solution of the diazo compound. These findings are in agreement with the earlier observations that diazo compounds or the product of coupling with gelatin are antiseptic (4).

Effect of Azoproteins on Rabbit Fibroma (Shope)

The results given in Table II were recorded 6 to 7 days after the injection of the infective material into the rabbit skin.

It will be seen from the table that injections of 1 cc. of the fibroma extract diluted with saline gave rise to small tumors, 2 to 2.5 cm. in diameter. The same amount of virus to which azoprotein solution had been added, produced extensive tumor formation. These were large masses of solid growth surrounded by a few scattered nodules. A typical example of this result is shown in Fig. 3. The injection of azoprotein and India ink in the same animal (Fig. 4) shows that the area of spread and the size of the tumor are practically the same.

TABLE III
Effect of Azoproteins on the Size of Skin Tumors in Chickens

Experiment No.	Azoprotein prepared from sulfanilic acid and	Apparent area of spread		Enhancement of tumors			Aspect of tumor on azoprotein side
		Saline (control)	Azoprotein solution	Age of tumors	Average size of tumors		
					Saline (control)	Azoprotein solution	
		sq.cm.	sq.cm.	days	sq.cm.	sq.cm.	
1	Egg albumin (3% sol)	3.2	25.5	16	7.3	44.8	Solid tumor surrounded by scattered nodules
2	Horse serum " "	3.7	19.2	21	4.0	16.4	Massive tumor
		4.1	55.1	21	3.9	49.6	Solid mass plus small nodules scattered around main tumor
3	" "	3.5	39.9	16	5.8	36.8	Solid tumor
4	" "	2.5	36.0	17	6.4	57.8	" "
5	" " " "			15	6.0	24.0	Scattered nodules only
				15	6.1	30.4	" " "

Effect of Azoproteins on Chicken Tumors

The effect of testicular extract on the development of skin tumors in chickens has been described in a previous paper (9). Table III shows the results obtained when azoprotein solutions were added to the tumor material before injection. As in the preceding cases, azoprotein solutions caused the tumor agent to spread over a large area of the skin, giving rise to large, massive tumors when the tumor

extract was very active, and to widely scattered nodules when the extract had a low tumor producing activity. The diffusion of the azoprotein solution in the chicken skin could be observed without the addition of India ink as an indicator. There was found to be a good agreement between the extent of the spread and the size of the tumors. Figs. 5 and 6 show tumors produced by a highly active extract and azoproteins, 16 days after the injection. Fig. 7 shows the effect of dispersion on material of low tumor producing activity.¹

SUMMARY

It is known that azoprotein solutions, like testicular extracts, possess the property of causing particles to spread through the dermis. The present work shows that azoproteins exhibit, like testicular extract, the power to increase the size of virus lesions in the skin of rabbits, and the size of tumors in chickens. The results indicate that the extent of the lesion is roughly proportional to the spreading power of the solution. This suggests that the spread of the infective material, over a larger area of skin, is directly responsible for the enhancing effect.

The production of extensive lesions by means of spreading agents may have a practical value when large amounts of working material are needed.

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¹ In addition to the above tests, the effect of azoproteins was tried on mouse and rabbit tumors. Duran-Reynals has shown that testicular extract mixed with the tumor cells would inhibit the development of Brown-Pearce tumors in the skin of rabbits (10). Azoproteins had no such effects on the growth of mouse tumor 48 or on the Brown-Pearce tumor. On the contrary a slight enhancing effect on the growth of these tumors was observed by Dr. Van der Schueren in this laboratory. However, it was noted that, when an azoprotein solution was mixed with the tumor cells, the material turned into a soft gel, and it is possible that this condition interfered with the usual spreading property of the compound.

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EXPLANATION OF PLATES

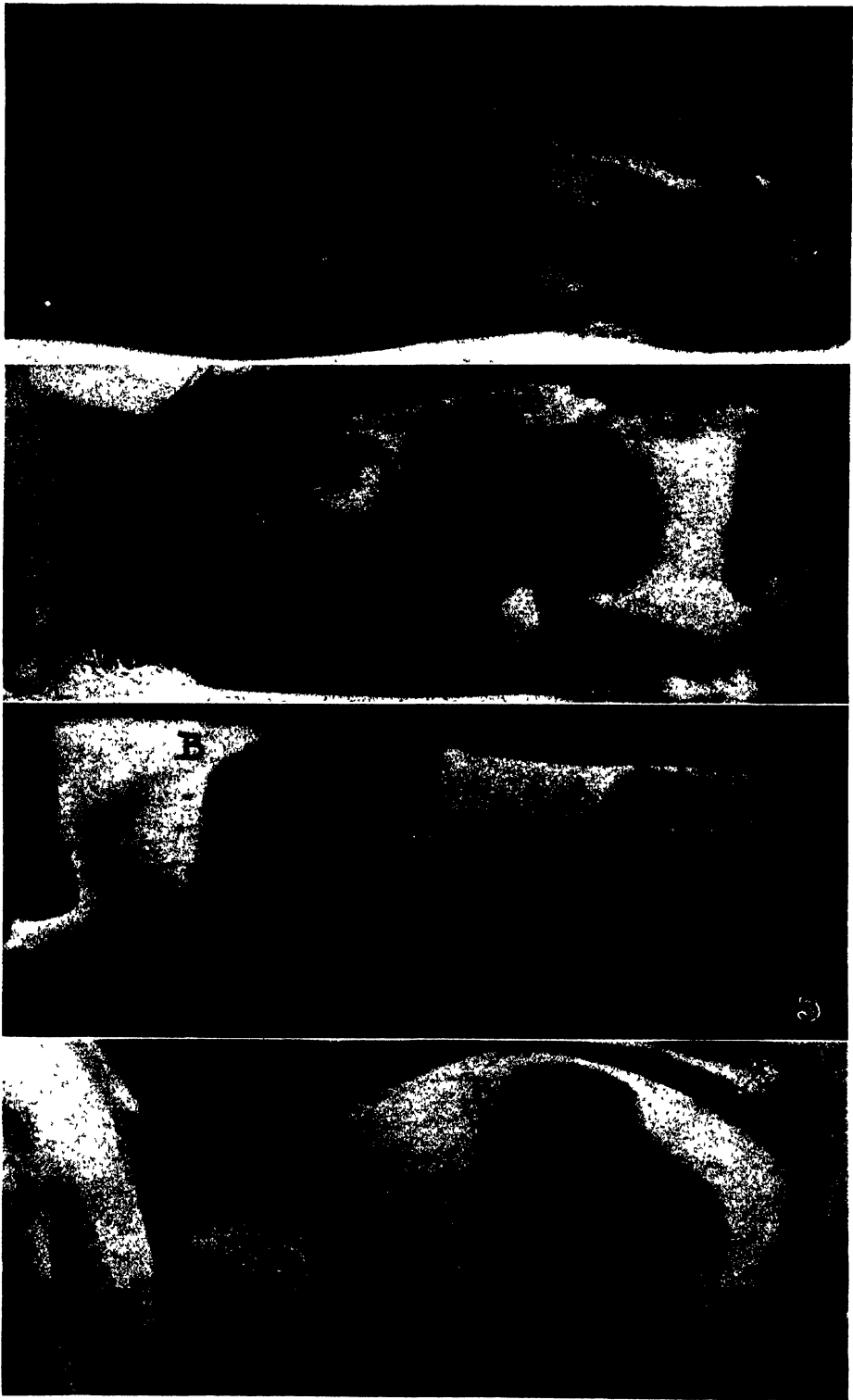
PLATE 38

FIG. 1. Rabbit 9-50. Lesions produced by the intradermal injection of 0.5 cc. vaccine virus suspension mixed with: A, 0.5 cc. saline solution (control) and B, 0.5 cc. azoprotein solution prepared from egg albumin.

FIG. 2. Rabbit 9-49. Spread produced by intradermal injection of 0.5 cc. India ink suspension mixed with: A, 0.5 cc. saline solution (control) and B, 0.5 cc. azoprotein solution prepared from egg albumin. The area of spread, 24 hours after injection, was 87.0 sq. cm. for the azoprotein solution and 5.5 sq. cm. for the saline control.

FIG. 3. Rabbit 9-21 (right side). Skin fibroma produced by the intradermal injection of 0.5 cc. suspension of Shope fibroma virus mixed with: A, 0.5 cc. saline solution (control) and B, 0.5 cc. azoprotein solution prepared from horse serum.

FIG. 4. Rabbit 9-21 (left side). Spread produced by intradermal injection of 0.5 cc. of an India ink suspension mixed with an equal volume of: A, saline solution (control) and B, azoprotein solution prepared from horse serum. The area of spread 24 hours after injection was 7.5 sq. cm. for the control and 77.0 sq. cm. for the azoprotein solution.



Photographed by Joseph B Haulenbeek

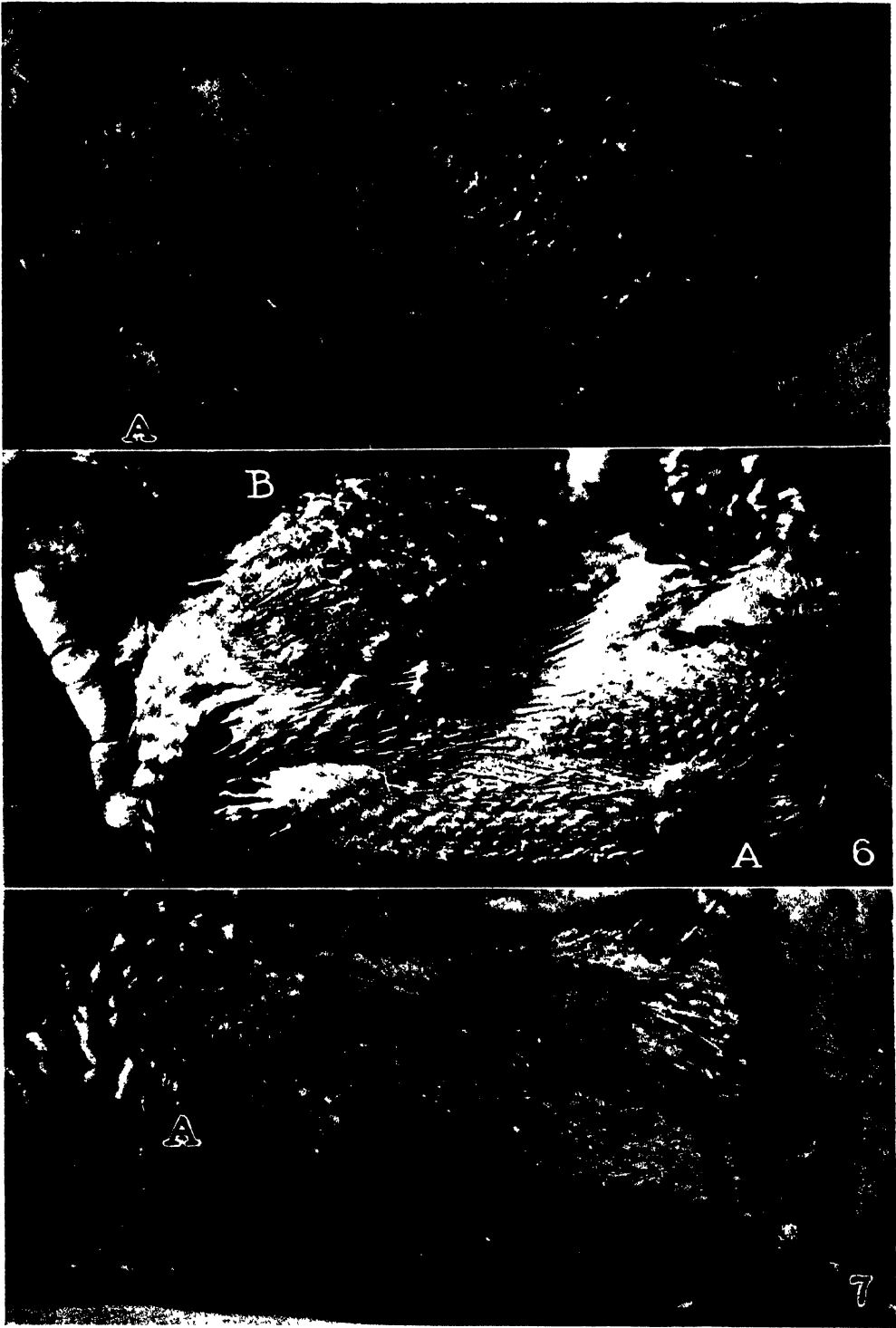
(Claude: Enhancing effect of azoproteins on lesions)

PLATE 39

FIG. 5. Chicken 8-84. Skin tumors produced by intradermal injection of 0.4 cc. chicken tumor extract mixed, in the syringe, with an equal volume of: A, saline solution (control) and B, azoprotein solution prepared from horse serum. The size of the tumor, recorded 16 days after injection, was 2.2×2.1 cm. for the saline control and 7.7×6.8 cm. for the azoprotein mixture.

FIG. 6. Chicken 8-73. Skin tumors produced by intradermal injection of 0.4 cc. chicken tumor extract mixed with: A, saline solution (control) and B, azoprotein solution prepared from horse serum. 21 days after injection there was a main tumor, 6.5×6.5 cm. at the site of injection, surrounded by smaller nodules scattered over an area 9.2×6.7 cm. The tumor produced by the saline mixture measured 2.2×2.1 cm.

FIG. 7. Chicken 8-69. Skin tumors produced by intradermal injection of 0.4 cc. chicken tumor extract mixed with: A, 0.5 cc. saline solution (control) and B, 0.5 cc. azoprotein solution prepared from horse serum. In this case, the tumor extract was not very active and the lesions produced by the azoprotein mixture were represented by small isolated tumors scattered over an area 9.0×4.7 cm. The control mixture produced a solid tumor, 2.8×2.7 cm. across.



Photographed by Joseph B Haulenbeek

(Claude: Enhancing effect of azoproteins on lesions)

ON THE SEROLOGICAL SPECIFICITY OF PEPTIDES. III

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For the purpose of studying the serological behavior of synthetic substances of somewhat complex structure, which at the same time bear a relation to natural antigens, we have proceeded to investigate immune sera to azoproteins made from longer peptide chains than before (1, 2), that is, pentapeptides; the latter again were built up from glycine and *d,l*-leucine. Since in previous studies the terminal amino acid, with free carboxyl group, dominated to a considerable extent in the serological reactivity, peptide amides have now been included in order to eliminate the prominent influence of acid groups and to get information on the rôle played by other parts of the molecule in the reactions with immune bodies. The peptides used for the preparation of azo antigens were tetraglycyl-glycine, tetraglycyl-leucine, *d,l*-diglycyl-leucyl-glycyl-glycine and trileucyl-glycyl-glycine.¹

EXPERIMENTAL

The preparation of a number of the substances used has been described previously (1, 2). With some of these compounds, namely the first four mentioned below, more convenient methods have now been utilized. Melting points were not corrected and mostly no attempt was made to recrystallize to constant melting point.

p-Nitrobenzoyl-Tetraglycyl-*d,l*-Leucine.—7.4 gm. of *p*-nitrobenzoyl glycyl-glycine hydrazide (see below) were dissolved in 15 cc. 5/*N* HCl, 100 cc. of 50 per cent acetic acid, and 150 cc. of water, and were converted into the azide at 0–5°C. by slow addition of a solution of 2.6 gm. of sodium nitrite in 10 cc. of water. After ½ hour at 0–5°C. the azide was filtered off and washed with cold water until

¹ These compounds are hereafter designated respectively G₆, G₄L, G₂LG₂, L₃G₃, and the amides by the symbol Am. The preparation trileucyl-glycyl-glycine may well be a mixture of steric isomers although the nitrobenzoyl derivative was crystalline. (For other abbreviations see references 1, 2.)

free from acid. The azide was dissolved in 250 cc. of cold 85 per cent alcohol and the solution was added to a neutral solution of 7.4 gm. of diglycyl-*d,l*-leucine in 300 cc. of water. The mixture was stirred and 40 cc. of $N/NaOH$ were added in small portions over a period of 1 hour to keep the solution slightly alkaline to litmus. After 2 more hours the solution was made weakly acid to litmus and concentrated *in vacuo* at 40° to a volume of 150 cc. If the solution became alkaline to litmus during the distillation, it was neutralized with HCl. After removal of a small amount of alkali insoluble material by filtration, the solution was made acid to Congo red. The sticky precipitate crystallized upon rubbing (needles). Yield 7.5 gm. It was recrystallized from water and again from 60 volumes of 50 per cent alcohol. Bushels of needles, m.p. unsharp with decomposition at 240°C. Analysis: Calculated for $C_{21}H_{38}O_9N_6$: N 16.54, found 16.49.

p-Aminobenzoyl-Tetraglycyl-*d,l*-Leucine.—The nitro compound was dissolved in 50 volumes of 75 per cent alcohol and reduced by means of palladium black and hydrogen at atmospheric pressure. The solution was evaporated to dryness *in vacuo* and the amino compound was recrystallized from 50 per cent alcohol. Microcrystalline. Yield 65 per cent. M.p. 165–166°C. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.50.

p-Nitrobenzoyl-Diglycyl-*d,l*-Leucyl-Glycyl-Glycine.—This substance was prepared by the above method using 7.4 gm. of *p*-nitrobenzoyl-glycyl-glycine hydrazide and 7.4 gm. of *d,l*-leucyl-glycyl-glycine. After completion of the reaction and concentration *in vacuo* the solution was made acid to Congo red by addition of HCl and kept overnight in the ice box. The crystalline precipitate (needles) was filtered and recrystallized from 100 cc. of water. Yield 6 gm. M.p. 144–145°C. Analysis: Calculated for $C_{21}H_{28}O_9N_6$: N 16.54, found 16.61.

p-Aminobenzoyl-Diglycyl-*d,l*-Leucyl-Glycyl-Glycine.—The nitro compound was reduced by means of palladium and hydrogen as described, the alcoholic solution was evaporated to dryness *in vacuo*, the amino compound was dissolved in a small amount of methyl alcohol and precipitated by addition of dry ether. The precipitate became granular upon rubbing with more dry ether. Yield 90 per cent. Analysis: Calculated for $C_{21}H_{30}O_7N_6$: N 17.58, found 17.41.

Esters and Amides of Nitrobenzoyl Amino Acids and Nitrobenzoyl Peptides.—The nitrobenzoyl compounds (1,2) were converted into methyl esters by either dissolving or suspending the finely ground substance in 5 parts of absolute methyl alcohol and adding in portions an ether solution of diazomethane at 0–5°C., using a slight excess. The mixtures were allowed to stand at room temperature and after removal of the solvents the substances were further purified as described below. The esters were changed into amides by dissolving them in 100 parts (or more) of hot absolute methyl alcohol and saturating the solutions with dry ammonia gas at 0–5°C. The solutions were kept in a closed flask at room temperature for 48 hours and were then evaporated to dryness *in vacuo*; the last traces of ammonia were removed by again adding dry methyl alcohol and evaporating to dryness. The amides were further purified by recrystallization.

Reduction of Nitrobenzoyl Compounds.—The aminobenzoyl amino acid amides and aminobenzoyl peptide amides were prepared by suspending the finely ground nitro compounds in 50 volumes of 75 per cent ethyl alcohol (some were completely and others only partly dissolved) and reducing by means of palladium black and hydrogen at atmospheric pressure. After complete reduction no undissolved substance was left. The solutions were filtered and evaporated to dryness *in vacuo*. The amino compounds were further purified as described below.

p-Nitrobenzoyl-Glycine Ethyl Ester.—This was prepared by nitrobenzoylation of glycine ethyl ester by the method used previously for *p*-nitrobenzoyl-tyrosine ethyl ester (3). The chloroform solution of the nitrobenzoyl compound was evaporated to dryness *in vacuo* and the substance was freed from nitrobenzoic acid by dissolving in a small amount of hot chloroform and precipitating with 5 volumes of ether. It was recrystallized from 5 parts of absolute ethyl alcohol. Narrow platelets. Yield 16 gm. from 14 gm. of glycine ethyl ester hydrochloride. M.p. 141–143°C. Analysis: Calculated for $C_{11}H_{13}O_5N_2$: N 11.11, found 11.05.

p-Nitrobenzoyl-Glycine Amide.—Recrystallized from 30 parts of 80 per cent ethyl alcohol. Platelets. M.p. 239–240°C. Yield 80 per cent. Analysis: Calculated for $C_9H_9O_4N_2$: N 18.83, found 18.66.

p-Aminobenzoyl-Glycine Amide.—Recrystallized from 80 parts absolute methyl alcohol. Platelets. M.p. 225–226°C. Yield 70 per cent. Analysis: Calculated for $C_9H_{11}O_2N_3$: N 21.76, found 21.82.

p-Nitrobenzoyl-d,l-Leucine Methyl Ester.—Recrystallized from 20 parts of 50 per cent ethyl alcohol. Needles. M.p. 83–84°C. Yield 72 per cent. Analysis: Calculated for $C_{14}H_{18}O_5N_2$: N 9.52, found 9.48.

p-Nitrobenzoyl-d,l-Leucine Amide.—Recrystallized from 25 parts of 80 per cent ethyl alcohol. Platelets. M.p. 197–198°C. Yield 80 per cent. Analysis: Calculated for $C_{13}H_{17}O_4N_3$: N 15.05, found 14.86.

p-Aminobenzoyl-d,l-Leucine Amide.—Recrystallized from water. Platelets. M.p. 192–193°C. Yield 70 per cent. Analysis: Calculated for $C_{13}H_{19}O_2N_3$: N 16.87, found 17.05.

p-Nitrobenzoyl-Glycyl-Glycine Methyl Ester.—Recrystallized from 40 parts of 95 per cent ethyl alcohol. Platelets. M.p. 194–195°C. Yield 90 per cent. Analysis: Calculated for $C_{12}H_{15}O_6N_3$: N 14.24, found 14.36.

p-Nitrobenzoyl-Glycyl-Glycine Amide.—A solution of *p*-nitrobenzoyl-glycyl-glycine methyl ester in 150 parts of dry methyl alcohol was saturated with dry ammonia gas first at room temperature and subsequently at 0–5°C. From here on the general procedure was followed. Recrystallized from 120 parts of 80 per cent ethyl alcohol. Long needles. Upon rapid heating m.p. 257–258°C. with decomposition. Yield 80 per cent. Analysis: Calculated for $C_{11}H_{13}O_5N_4$: N 20.0, found 19.98.

p-Nitrobenzoyl-Glycyl-Glycine Hydrazide.—10 gm. of finely ground *p*-nitrobenzoyl-glycyl-glycine methyl ester were suspended in 100 cc. of absolute ethyl alcohol and 20 cc. of 100 per cent hydrazine hydrate were added. After shaking for 1 hour the mixture became very thick and the crystal form of the substance

had changed from the long platelets of the ester to hair-like needles of the hydrazide. The hydrazide was filtered off, washed with absolute alcohol, and recrystallized from 140 parts of 30 per cent ethyl alcohol. Needles. M.p. 250–251°C. Yield almost quantitative.

p-Aminobenzoyl-Glycyl-Glycine Amide.—Recrystallized from 80 parts of absolute methyl alcohol. Platelets. M.p. 211–212°C. Yield 70 per cent. Analysis: Calculated for $C_{11}H_{14}O_3N_4$: N 22.40, found 22.52.

p-Nitrobenzoyl-Glycyl-d,l-Leucine Methyl Ester.—Recrystallized from 50 per cent ethyl alcohol. Needles. M.p. 155–156°C. Yield 80 per cent. Analysis: Calculated for $C_{16}H_{21}O_6N_3$: N 11.97, found 12.20.

p-Nitrobenzoyl-Glycyl-d,l-Leucine Amide.—Recrystallized from 10 parts of absolute ethyl alcohol and also from ethyl acetate containing 10 per cent alcohol. Irregular platelets. M.p. 178–179°C. Analysis: Calculated for $C_{15}H_{20}O_5N_4$: N 16.67, found 16.70.

p-Aminobenzoyl-Glycyl-d,l-Leucine Amide.—Recrystallized from 20 parts of water. Needles. Yield 80 per cent. Analysis: Calculated for $C_{15}H_{22}O_3N_4$: N 18.30, found 18.18.

p-Nitrobenzoyl-Diglycyl-d,l-Leucine Methyl Ester.—Recrystallized from 20 parts of 50 per cent methyl alcohol. Needles. M.p. 177–178°C. Yield 70 per cent. Analysis: Calculated for $C_{18}H_{24}O_7N_4$: N 13.72, found 13.67.

p-Nitrobenzoyl-Diglycyl-d,l-Leucine Amide.—Recrystallized from 20 parts of 60 per cent ethyl alcohol. Platelets. M.p. 198–200°C. Yield 80 per cent. Analysis: Calculated for $C_{17}H_{23}O_6N_5$: N 17.81, found 17.95.

p-Aminobenzoyl-Diglycyl-d,l-Leucine Amide.—The substance was dissolved in a small amount of absolute methyl alcohol and precipitated from this solution by addition of 10 volumes of dry ether. Amorphous. Shrinks at 137°C. Yield 80 per cent. Analysis: Calculated for $C_{17}H_{25}O_4N_5$: N 19.28, found 19.38.

p-Nitrobenzoyl-d,l-Leucyl-Glycyl-Glycine Methyl Ester.—Recrystallized from 60 parts of 30 per cent ethyl alcohol. Platelets. M.p. 154–155°C. Yield 90 per cent. Analysis: Calculated for $C_{18}H_{24}O_7N_4$: N 13.72, found 13.55.

p-Nitrobenzoyl-d,l-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 40 parts of 95 per cent ethyl alcohol. Needles. M.p. 201°C. Yield 75 per cent. Analysis: Calculated for $C_{17}H_{23}O_6N_5$: N 17.81, found 17.62.

p-Aminobenzoyl-d,l-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 15 parts of absolute methyl alcohol. Platelets. M.p. 170–171°C. Yield 70 per cent. Analysis: Calculated for $C_{17}H_{25}O_4N_5$: N 19.28, found 19.18.

p-Nitrobenzoyl-Tetraglycyl-d,l-Leucine Methyl Ester.—Recrystallized from 30 parts of 50 per cent ethyl alcohol. Hair-like needles. M.p. 243–244°C. Yield 90 per cent. Analysis: Calculated for $C_{22}H_{30}O_9N_6$: N 16.09, found 16.05.

p-Nitrobenzoyl-Tetraglycyl-d,l-Leucine Amide.—*p*-Nitrobenzoyl-tetraglycyl-*d,l*-leucine methyl ester was dissolved in 300 parts of boiling dry methyl alcohol and the solution was saturated with dry ammonia gas first at room temperature and subsequently at 0–5°C. Then the general procedure was followed. Re-

crystallized from 30 parts of 50 per cent ethyl alcohol. Small needles. Yield 85 per cent. Analysis: Calculated for $C_{21}H_{29}O_8N_7$: N 19.35, found 19.24.

p-Aminobenzoyl-Tetraglycyl-*d*,*l*-Leucine Amide.—Recrystallized from 30 parts of absolute methyl alcohol. No distinct crystalline form. Shrinks at 180°C. and melts at about 185°C. Yield 85 per cent. Analysis: Calculated for $C_{21}H_{31}O_8N_7$: N 20.54, found 20.40.

p-Nitrobenzoyl-Diglycyl-*d*,*l*-Leucyl-Glycyl-Glycine Methyl Ester.—Recrystallized from water. Rosettes of needles. Yield 75 per cent. Analysis: Calculated for $C_{22}H_{30}O_9N_6$: N 16.09, found 15.98.

p-Nitrobenzoyl-Diglycyl-*d*,*l*-Leucyl-Glycyl-Glycine Amide.—Recrystallized from 20 parts of 50 per cent ethyl alcohol. Hair-like needles. M.p. 216–217°C. Yield 80 per cent. Analysis: Calculated for $C_{21}H_{29}O_8N_7$: N 19.35, found 19.18.

p-Aminobenzoyl-Diglycyl-*d*,*l*-Leucyl-Glycyl-Glycine Amide.—For purification the substance was dissolved in 15 parts of hot amyl alcohol. Amorphous substance which separated on cooling was filtered off and washed with dry ether. Yield 70 per cent. Analysis: Calculated for $C_{21}H_{31}O_8N_7$: N 20.54, found 20.38.

Azodyes.—The dyes used for inhibition tests were prepared by coupling the diazonium compounds, for $\frac{1}{2}$ hour at 0–5°C., with an equimolar quantity of *m*-hydroxybenzoic acid in a solution kept alkaline by addition of a slight excess of sodium carbonate. After acidification and centrifugation the dyes were redissolved by means of dilute NaOH and after determination of the contents of dye stock solutions were made up to a concentration of 1:4 millimol of dye in 10 cc.

Immunization.—Rabbits were injected intravenously with 5 mg. of a suspension of horse azostromata (4) in 2 cc. After two to four courses of 6 daily injections each, with rest intervals of 1 week, the animals were bled on the 7th day following the last injection.

Tests.—Antigens used for the tests were made with chicken serum as described (2) using half as much of the diazonium compounds. The dilutions of the test antigens given in the tables are in terms of a 5 per cent stock solution. The tests were observed for 1 hour at room temperature and then kept overnight in the ice box. The intensity of the reactions is indicated as follows: 0, f. tr. (faint trace), tr. (trace), $\overline{\text{tr.}}$ (strong trace), \pm , $+$, $++$, $+++$, etc.

In the inhibition tests, the appropriate amount of the solutions had to be determined by preliminary experiments.

Precipitin and Absorption Tests

The precipitin reactions of immune sera for the four pentapeptides are presented in Tables I and II; they are in keeping with those exhibited by sera to shorter peptide chains in that cross reactions occurred chiefly, but not exclusively (*e.g.* G₄L serum No. 2 on G₄ or on LG₄) with peptides having the same amino acid at the carboxyl

end. The cross reactions were definitely related to similarities in constitution. For instance, the L_3G_2 immune serum gave precipitation with G_2 , not with LG or GLG and the reactions of G_4L immune serum No. 2 increased in strength in the sequence L, GL, G_2L , etc. Or, G_5 immune serum precipitated G_2 but not LG and, G_3 much more than LG_2 .

The antisera for G_4L Am. distinguished sharply between G_4L Am. and G_2LG_2 Am. (Table II) although they differ only in the

TABLE III

To 0.04 cc. of the 1:100 diluted antigens were added 0.16 cc. absorbed immune serum (I.S.).

Two absorptions were carried out with G_5 azostromata, using 1.4 mg. with 1 cc. serum. With LG_2 the first absorption was made similarly, the second with half as much azostromata. The readings made after 15 minutes are shown in the first line, and those after 1 hour in the second line.

	G_2	LG_2	G_5	G_2LG_2
G_2LG_2 I.S. No. 1 absorbed with G_5	tr. tr.	\pm +	0 0	++ ++±
G_2LG_2 I.S. No. 1 absorbed with LG_2	\pm ±	0 0	+ ±±	++ +++

position of the leucine residue in the chain. Furthermore, the peptides and their corresponding amides in spite of great similarity in structure proved to be serologically different; G_4L and G_4L Am. showed weak, G_2LG_2 and G_2LG_2 Am. marked overlapping reactions. In addition cross precipitations were seen among amides as noted for peptides, however there was little overlapping between the two sorts of compounds. Of particular interest are observations that G_2LG_2 Am. sera react only faintly at best with LG_2 Am. antigen, which is identical as to the terminal part of G_2LG_2 Am., but do react (as to a lesser extent do G_2LG_2 sera) with G_2L Am., G_4L Am., and GL Am., which correspond to interior portions of the homologous substance. This is in contrast to the marked determinant influence of acid groups regularly encountered with free peptides, and the conclusion may be drawn that amide groups, though also strongly polar

(5), differ considerably from free acidic groups with regard to the effect on serological reactivity.

TABLE IV

The tests were put up as described in Table III. Two absorptions were made of G_2LG_2 immune serum, each time with 2.7 mg. G_5 azostromata and 1 cc. serum; for comparison, similar tests were made of the unabsorbed immune serum diluted with normal rabbit serum (see sub-table, readings after an hour). The L_3G_2 immune serum (1 cc.) was twice absorbed with G_3 (rabbit) azostromata, first with 0.7 mg. stromata, then with half as much. The reactions of the unabsorbed sera, although not directly comparable, are taken from Table I to indicate their relative strength.

The readings shown in the table were made after 1 hour and overnight in the ice box.

	G_1	G_2	G_3	G_4	G_5	LG_2	G_2LG_2	L_3G_2	LG_4	Glut. G_2	Tyr. G_2	Glutathione
G_2LG_2 I.S. No. 1 abs. with G_5	0 0	0 0	0 0	0 0	0 0	f. tr. tr.	++ ++±	0 0	0 0	0 0	0 0	0 0
Unabsorbed	tr.	+	++	++	++	++	+++	+	±±	+	±	0
L_3G_2 I.S. abs. with G_3		0 0	0 0	0 0	0 0	± ±±	± ±±	±± ±±±	0 0	0 0	0 0	
Unabsorbed		+	±±	+	+	±±	±±	±±	+	±	f. tr.	

	G_3	LG_2	G_5	G_2LG_2
G_2LG_2 I.S. No. 1 Diluted 1:2	++	±±	++	++±
Diluted 1:4	+	±	±	±±
Diluted 1:8	tr.	0	f. tr.	tr.

Previous experience with the complexity of immune sera (4) suggested absorption experiments which were carried out on a limited scale, using, as before, insoluble antigens (azostromata). With one serum each for G_5 and G_4L no clear-cut separation of antibody fractions was obtained by treatment respectively with azostromata G_5 and G_2L ; but antibodies with different specificities actually were

demonstrable in G_2LG_2 sera on absorption with G_5 and LG_2 stromata (Table III); a similar result was observed when G_4L immune sera were absorbed with G_5 and G_4L Am.

The effect of partial absorption with heterologous peptide azostromata on the cross reactions of L_3G_2 and G_2LG_2 sera is seen from Table IV. With suitably absorbed serum L_3G_2 the supernatant fluid reacted only with the peptides containing a terminal LG_2 and with none of the other peptides. A still higher degree of specificity ap-

TABLE V

To 0.2 cc. of 1:500 dilutions of the antigens were added 3 capillary drops of immune serum or absorbed serum prepared by absorption for 2 hours at room temperature with 2 mg. of G_2LG_2 per cc. or (for control of non-specific absorption) G-azostromata. The readings given were made after 1 hour and overnight in the ice box.

Antigens	Immune serum for G_2LG_2 amide		
	Unabsorbed	Absorbed with G	Absorbed with G_2LG_2
G_2LG_2	++	++	0
	++±	++±	0
G_2LG_2 amide	++±	++±	0
	+++	+++	tr.

peared from the tests with immune sera G_2LG_2 : absorption with G_5 in increasing amounts removed the cross reactions stepwise, and finally, using a proper amount of adsorbant, antibodies were left behind that were specifically adjusted to the entire structure (azobenzoyl) G_2LG_2 and did not act on the other related peptide antigens except for a slight reaction with LG_2 . The control experiment, as tabulated, showed that the result is not due merely to diminution of the antibody content, as would be brought about by simple dilution.

As regards the overlapping reactions between peptides and corresponding amides (see Table II), the objection could be raised that the amides might have been hydrolyzed to some extent in the animal body with production of some free acid and antibodies thereto or, on the other hand, that such splitting occurred during the preparation of the azoantigens, resulting in a mixed antigen such as could give

rise to reactions with sera for the free peptides. However, these possibilities are ruled out by the following experiments. A serum for G₂L G₂ Am. was adsorbed with G₂L G₂ azostromata, and for control purposes with a non-reacting antigen as well, with the result that the antibodies which reacted with the amide were almost com-

TABLE VI

To 0.2 cc. of the given dilutions of stock antigen was added 1 capillary drop (in the case of G₄L amide antigen 3 drops) of immune serum either unabsorbed and diluted with normal rabbit serum or after being absorbed with G₄L Am. (2 mg. stromata per 1 cc. serum; 2 hours at room temperature). The readings given were made after 1 hour and after the tests had stood overnight in the ice box.

Immune serum G ₄ L (No. 2)	G ₄ L antigen						G ₄ L Am. antigen					
	1:50	1:100	1:200	1:400	1:800	1:1600	1:50	1:100	1:200	1:400	1:800	1:1600
After absorption with G ₄ L amide	0	±	+±	++±	++±	++	0	0	0	0	0	0
	0	±	++	++±	++±	++	f. tr.	f. tr.	f. tr.	0	0	0
Unabsorbed; diluted 3:4 in normal rab- bit serum	tr.	±	+±	++	++	+±		+				
	tr.	+	++	++±	++±	++		+±				
Unabsorbed; diluted 1:2 in normal rab- bit serum	f. tr.	±	+	+±	+±	+		+				
	tr.	±	+	++	++±	+±		+				

pletely exhausted by the peptide antigen (Table V). (With another serum only a small amount of antibodies was left behind.) This proves that the bulk and not only a minor portion of the antibodies contained in the amide serum reacts with the free peptide. Then in the case of G₄L immune serum (Table VI) the reactions with G₄L Am. antigen disappeared almost completely upon absorption with G₄L Am. stromata, while the homologous reaction was hardly diminished and this, taken in conjunction with the persistence of the reaction for G₄L Am. upon dilution of the unabsorbed G₄L immune serum, shows that in the G₄L Am. antigen there is no appreciable amount of G₄L and that the sera contain a small fraction of a special antibody cross reacting with the amide.

TABLE VII *a*

For the inhibition tests 0.05 cc. of neutral solutions of nitrobenzoyl peptides were mixed with 0.2 cc. of 1:500 dilutions of the stock chicken antigens, and homologous immune sera were then added (2 drops in the case of G_5 and 3 drops of G_4L and G_2LG_2); the concentrations of the inhibiting solutions (millimols in 10 cc.) were 1:8 for serum G_4L , 1:2 for serum G_2LG_2 , and 1:4 for serum G_5 . The control tube contained only antigen and immune serum.

Readings taken after 1 hour and after standing overnight.

	L	GL	G_2L	G_3L	G_5	G_4L	G_2LG_2	Control
G_4L I.S. (No. 4)	+	+	tr.	tr.	++	0	++	++
	++	++	+	±	+++±	tr.	+++	+++±

	G_2	G_3	LG_2	G_4	G_5	LG_4	G_2LG_2	G_4L	Glut. G_2	Tyr. G_2	Control
G_5 I.S.	+	0	+	0	0	0	+	+	+	±	++
	±	±	±	tr.	0	tr.	±	±	++	++	+++
G_2LG_2	+	+	tr.	+	+	+	0	±	+	±	++
I.S. (No. 3)	+++	+++	++	+++	+++	+++	0	+++±	+++	+++	+++±

TABLE VII *b*

For the inhibition tests 0.05 cc. of solutions of peptide amide azodyes (concentration 1:33 millimol in 10 cc.) were mixed with 0.2 cc. of 1:500 dilutions of the stock G_4L Am. chicken antigen, and 2 capillary drops of immune serum were then added. Readings are given after 15 minutes and 1 hour, and after standing overnight.

	G Am.	L Am.	G_2 Am.	GL Am.	G_2L Am.	LG_2 Am.	G_4L Am.	G_2LG_2 Am.	G_4L	Control
G_4L amide I.S.	±	+	±	±	±	±	0	±	+	±
	++	±	++	+	+	++	tr.	++	±	++
	+++	+++	+++	±	±	+++	tr.	+++	+++	+++

Inhibition Tests

Inhibition tests were carried out with nitrobenzoylated peptides;² peptide amides because of their poor solubility were converted into

² Positive inhibition reactions were also obtained with higher concentrations of non-acylated G_2LG_2 . The compounds G_5 , L_3G_2 , G_4L , NO_2 -benzoyl L_3G_2 could not be tested because of insufficient solubility.

easily soluble azodyes by diazotizing the aminobenzoyl derivatives and coupling to *m*-hydroxybenzoic acid. Results are presented in Tables VII *a* and VII *b*. When it is taken into account that only those substances are included which in the form of azoproteins gave positive precipitin reactions with the sera in question, these tests are

TABLE VIII

For the inhibition tests neutral solutions of nitrobenzoyl peptides were mixed with 0.2 cc. of 1:500 dilutions of the heterologous antigens before addition of the immune sera. In the case of G₄L serum, 0.05 cc. of solutions containing 1:8 millimol of the peptides in 10 cc. were tested, with G₂LG₂ serum, 0.1 cc. of solutions containing 1:12 millimol in 10 cc. 2 drops were used of G₄L and 3 drops of G₂LG₂ immune sera.

Readings taken after 1 hour and after standing overnight.

	L	GL	G ₂ L	G ₅	G ₄ L	Control
G ₄ L I.S. No. 3 on G ₂ L antigen	+	±	tr.	+±	0	+±
	++	±	±	++±	tr.	++±

	G	G ₂	G ₂ L	LG ₂	G ₅	G ₂ LG ₂	LG ₄	Control
G ₂ LG ₂ I.S. No. 4 on G ₂ antigen	+	±	+	±	±	0	±	+
	+±	+	+±	+	+	tr.	+	+±

rather strikingly specific for the homologous haptens. The distinction between the sera G₅ and G₂LG₂ differing in only one amino acid, may be mentioned and, again, the shading off of the reactions of sera G₄L, in the order G₄L, G₃L, G₂L, GL, and L (Table VII *a*). The amide azodyes did not inhibit the reactions of the peptide immune sera but showed sharply specific inhibitions with the homologous amide sera (Table VII *b*). A definite reaction was also seen with G₂LG₂ Am. serum and G₂LG₂ used either as nitrobenzoyl derivative or as azodye.

The inhibition method proved to be of advantage for characterizing the nature of antibodies operative in the cross precipitin reactions with heterologous antigens.

From such experiments in part presented for illustration (Table VIII), it appears that also those antibodies that produce precipitation with heterologous antigens are specifically related to the homologous substance in its entirety (page 129), that is to the one which served as determinant in the formation of the antibodies. When, therefore, one antigen is precipitated by several different immune sera, it can be shown by inhibition tests that the antibodies concerned are different, as in the instances given in Table IX.

TABLE IX

The procedure for the inhibition tests was that described in Table VII a; the concentrations of the inhibiting solutions were (as millimols in 10 cc.) 1:2, 1:8, and 1:16 as employed respectively with the three immune sera listed. 2 drops each were used of G_5 and G_2LG_2 , and 3 drops of G_3 immune sera.

The readings presented were made after 15 minutes, 1 hour, and after standing overnight.

	G_3	G_5	G_2LG_2	Control
G_3 I.S. on G_3 antigen	0	tr.	+	++
	f. tr.	tr.	+	++±
	f. tr.	tr.	+±	++±
G_5 I.S. on G_3 antigen	tr.	0	+	+±
	+	tr.	+±	++
	+	±	++	++±
G_2LG_2 I.S. No. 2 on G_3 antigen	tr.	tr.	0	+
	+	±	0	+±
	+	±	0	+±

COMMENT

The four pentapeptides examined, although all contained only glycyl and leucyl residues, were distinct in their precipitin reactions when tested with various antigens; and replacement of even one of five glycines by leucine resulted in a noticeable alteration in serological properties. A pronounced serological change was brought about by the conversion of peptides into amides, and a similar modification was observed upon esterification of peptides (6).

The specificity of the sera was more conspicuous in inhibition than

in precipitin tests. The two kinds of reactions cannot readily be compared quantitatively, yet the apparently greater specificity of inhibition tests may be explained by the consideration that in inhibition reactions there is a competition between the weak affinity of the immune sera for heterologous haptens and the strong affinity to the homologous antigen, while in precipitin reactions there is present only one reacting substance and, moreover, precipitins of low activity can be aided and carried down in the precipitate by more potent antibodies (Heidelberger).³

From the absorption experiments described above one can conclude that the specificity of antibodies engendered by pentapeptides may be directed towards the molecule as a whole. In agreement with this, greater or at least equal inhibition of cross reactions was produced by the haptens homologous to the immune sera in comparison with those corresponding to the heterologous antigens tested (4). No evidence was found to demonstrate the formation of separate antibodies to several parts of the peptide structure, as had been observed with some other compounds in which highly determinant acid groups were linked to, and separated by a benzene ring (8). The difference in constitution between compounds of this sort and —CO—NH— chains may possibly be the reason for the disparity in the character of the antibodies formed. While thus it appears that antibodies may be formed which are specific for peptide chains consisting of five amino acid residues, it will be one of the next tasks to investigate, with the use of higher polypeptides, how large a structure in its entirety may be reflected in the configuration of antibodies, a question of considerable significance for the serological specificity of proteins.

The overlapping reactions of the peptide sera here reported were in general in accord with those formerly observed but a new fact emerged from the reactions of immune sera to a peptide amide (diglycyl-leucyl-diglycyl amide), namely strong cross reactions involving not the end groups but other parts of the molecule, a result likewise to be

³ The lesser specificity reported for inhibition reactions with very simple compounds, *e.g.* substituted benzoic acids (7), may be ascribable to the circumstance that the "homologous" haptens did not so closely correspond to the antigens used in which the azodye structure probably plays a greater part in determining the reactivity than in the more complicated instances.

considered in protein reactions. These sera, unlike those for the corresponding free peptide, gave no or only weak overlapping reactions with peptide amides identical in their terminal groups with the homologous substance. It would seem, therefore, that the serological predominance of carboxyl groups (see also Goebel, 9) is suppressed by conversion into amides and that there is a definite difference in the serological significance of strongly polar amides and of dissociating acid groups. This may suggest that the combinations occurring in antigen-antibody reactions are not all of the same kind.

SUMMARY

Experiments are described dealing with immune sera to pentapeptides and peptide amides. Absorption and inhibition tests gave no indication of the presence in the immune sera of special antibodies for portions of a peptide molecule but the antibodies appeared to be specific for an entire pentapeptide even though the sera contained qualitatively different fractions. Marked disparity was found between the reactions of peptides and corresponding amides indicating differences between acid and other polar groups in their influence on serological specificity.

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STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

VI. EXPERIMENTS ON THE SENSITIZATION OF GUINEA PIGS TO POISON IVY

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PLATE 42

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One of the open questions concerning skin sensitization of the "contact dermatitis" type to simple chemical compounds is in which way sensitivity spreads all over the integument when only a given area is treated. This subject has been considered in a paper by Simon (1) and studied experimentally. The skin of guinea pigs was treated with concentrated nitric acid in such manner that a complete belt severed the continuity of the epidermis. Poison ivy extract was applied on the posterior part of the animals and after 10 days the anterior and the posterior parts were tested; both halves were seen to be sensitive. According to the author the evidence "indicates that the route of distribution is not confined to the epidermis," and suggests that the spread is due to distribution of altered allergen by the blood stream or the lymphatic system.

Furthermore, Simon observed that no significant difference exists in the degree of sensitivity between the area used for the sensitizing application and other parts of the skin, and by timed excisions found that such removal of the treated area did not interfere with the general skin sensitization provided it was performed later than 18 to 24 hours after the application of ivy extracts. He also found that sensitivity was first seen-after a latency period of from 4 to 6 days.

In contrast to the result of Simon, Straus and Coca (2) reported that in monkeys severance of the continuity of the skin some distance above the elbow and application of poison ivy extract to the forearm

resulted in sensitization of this part only and prevented general skin sensitization. The experiment was also performed in reversed fashion. In the authors' opinion, their results suggest that the spread of sensitization is attributable "probably to a diffusion of the oily excitant through the oily substances normally present in the skin," in harmony with a hypothesis previously advanced by Coca (3, 2).

Similar results have recently been described in a preliminary communication by Schreus (4) who treated guinea pigs with dinitrochlorobenzene, a substance found in this laboratory (5, 6) to sensitize these animals. This author assumes spread by way of the intercellular bridges connecting the cells of the epidermis.

Further investigation of the subject was desirable, particularly in view of the lack of agreement in the reported experiments.

General Methods

Male albino guinea pigs from healthy stock, caged separately, were employed in the experiments; since operative procedures were to be carried out, rather heavy animals were commonly selected (500 to 600 gm. weight). For uniformity as regards the period allowed for sensitization (10 to 14 days) a group included only the animals operated upon within a 3 day period, and these were tested at the same time; in instances of comparisons between differing methods, the operations in question were done as far as practicable in alternation to avoid technical bias. All operations were conducted under deep ether anesthesia.

Sensitizing Application.—Poison ivy extract Lederle,¹ supplied as a 12½ or 13 per cent solution in acetone of extractives from *Rhus toxicodendron radicans*, was used to sensitize guinea pigs by application to the skin on a clipped (frequently also shaved) site, spread evenly over a circular area 10 to 11 mm. in diameter with the tip of a thin glass rod. The doses ranged from 0.05 cc. of the undiluted extract to 0.025 cc. of a 1:5 dilution in acetone or less, delivered as a series of micro drops (0.05 cc. = 10 to 13 drops) from a gauge 26 Luer needle with tip ground flat, attached to a 1 cc. Dewitt and Herz record (tuberculin) syringe where the amount discharged can be accurately controlled by a check-nut on the plunger shaft. Upon full evaporation of the solvent, an even, brownish, waxy film remained on the skin.

Rather elaborate precautions were taken in the case of experimental skin barriers to ensure that no ivy material could come into contact with other parts of the skin than the desired field. To this end, several layers of bandage gauze and an outer covering of crepe tissue paper were used to shield the body, the site

¹ This material came to us through the courtesy of Dr. Arthur F. Coca of the Lederle Laboratories. It may be recalled that the active principle of poison ivy is urushiol, a catechol with a fifteen carbon, unsaturated side chain (cf. 6).

of application being exposed through a hole in the coverings, which were firmly held against the skin, and likewise all instruments used after application of the ivy extract were not handled again until they had been cleaned chemically (with alcohol, and then with potassium permanganate). Finally the deposit was covered with a 14 mm. square of cellophane (cut from cellophane tubing, each of the two adhering layers being about 0.002 inch thick) cemented to the skin, along its margin, by means of duo liquid adhesive (Johnson and Johnson); over this was affixed a disc, about 30 mm. in diameter, of finely woven linen, the adhesive first being applied both to the periphery of the linen and to the skin outside the cellophane patch.

On the 4th or 5th day the ivy was removed, either, in early experiments, by cleaning the site with olive oil and acetone and painting with aqueous permanganate solution, or by excision, *in toto*, of the treated skin and its protective coverings, as described below.

Test for General Cutaneous Sensitivity.—On the 10th to 14th day following the original application of ivy extract to the skin, various areas were clipped with an electric clipper, and on these sites single drops (approximately 1/60 cc.) of freshly prepared dilutions in 95 per cent alcohol of the extract were allowed to fall from a fine capillary pipette. The drops were at once spread with the tip of a thin glass stirring rod over an area about 10 to 11 mm. in diameter and dried in a gentle current of air from a small electric blower; the sites were marked by touching the centers with the moistened tip of an indelible pencil.

The highest concentration of ivy causing no, or occasionally a slight reaction with normal animals was determined for each lot of extract (a 1:15 or 1:20 dilution), and was applied to the flank and to 2 sites on the back while areas close to these were tested with half the concentration (see Figs. 1, 2); and one-half and one-third concentration were used on the belly.

First readings were taken 24 hours later, some time after the sites had been cleaned with pledgets of cotton wet with acetone; second readings were made the day following. With the most sensitive animals, the reactions are strongest at 24 hours, but then turn brownish and begin to fade, while animals of lesser sensitivity may exhibit higher reactions at the second reading. In our experience, there was no difficulty in recognizing the difference between even moderately sensitized animals and normal controls similarly tested.

The intensity of the reactions was designated as follows: + + + +, pink or dark pink, sometimes slightly elevated; + + +, pink, but either somewhat pale or macular; + +, between faint pink and pale pink in color; +, faint pink; ±, faint pink ring; tr., trace; f.tr., faint trace.

EXPERIMENTAL

In order first to determine, under the conditions of our experiments, how long the active material must remain in contact with the skin to induce hypersensitiveness, and the length of the latency period before sensitivity appears, experiments similar to those of Simon (1; *cf.* 7)

were made. As regards the first question, sensitization was found to result if the treated area was extirpated later than 8 to 12 hours following the application (Table I).

TABLE I

Composite Table

Sensitization with poison ivy extract in relation to the excision of treated skin areas at different times (see text). The readings recorded were made 24 hours after application of the test doses on the 11th day; also observations at 48 hours are given (within parentheses) when there was an increase over the earlier reactions.

No.	Time before excision hrs.	Reactions to ivy extract			
		Dorsum		Flank	
		Dilutions		Dilutions	
		1:15	1:30	1:15	1:30
1	4	f.tr.	0	0	0
2	4	f.tr.	0	f.tr.	0
3	6	0	0	0	0
4	6	0	0	0	0
5	8	± (+++)	± (±)	± (++)	tr. (++)
6	8	0	0	0	0
7	12	++++	++	++++	+
8	12	++	+	+++	+++
9	16	++ (++++)	± (++)	± (++++)	± (+)
10	16	++++	++++	++++	++++
11	16	++++	+++	++++	+++
12	16	+ (++++)	tr. (±)	+ (++++)	± (+)
13	24	++++	+++	+++	+++
14	24	++++	+++	++++	+++
Normal controls					
15		0	0	tr.	0
16		f.tr.	f.tr.	0	0
17		f.tr.	f.tr.	tr.	0
18		± (tr.)	0	0	0
19		tr.	tr.	±	tr.
20		tr.	0	tr.	0

A linen ring having an outer diameter of about 26 mm. and an 18 mm. opening was affixed to the skin in the sacral region of the back by means of the liquid adhesive mentioned. The body of the guinea pig was shielded and only the skin

within the ring was exposed; on this site 0.03 cc. poison ivy extract was put on a circular area approximately 12 mm. in diameter. Upon evaporation of the acetone the residue was covered by a 14 mm. square of cellophane cemented at the corners to the skin and above it a linen disc 26 mm. in diameter was attached similarly to the linen ring. Cardboard collars (adapted from (8)) were fixed around the neck to keep the coverings undisturbed. After varying intervals the skin bearing the ivy and coverings was excised 1 to 2 mm. beyond the linen ring (with anesthesia); in this way not only the ivy-treated area but also a wide margin (8 to 11 mm.) outside was removed and the defect was dressed with thymol iodide. The animals were tested for sensitivity on the 11th or 12th day.

The somewhat shorter period of contact as compared with that of Simon possibly is due to the use of a larger dose of ivy extract or to some other technical difference. The necessary time of contact may be that required for absorption of a sufficient quantity of the incitant (which would vary according to such conditions as the amount and size of the treated area) or possibly an interval during which some chemical process takes place in the skin.

In our experiments on the latency period, hypersensitivity became manifest to tests made 5 days after the ivy was placed on the skin. Remarkably, the onset was quite regularly sudden with well developed reactions to the larger test dose present at the 24 hour reading and in tests made after 6 days the reactivity already was maximal (Table II).

The sudden appearance of hypersensitivity was further emphasized by the coincident and equal reactivities seen on the 6th day to the applications of the 5th day (one day reading) and the 4th day (48 hour reading), the latter having produced no significant reaction at 24 hours.

As pointed out before, the main issue of our investigation was rather the question whether an epidermal pathway is a necessary condition for the spread of sensitivity from one site to the whole body. In the first experiments, an area of skin on the back was isolated during deep anesthesia by means of a circular cautery burn roughly 5 mm. wide and deep enough to destroy the epidermis with certainty (*cf.* 1), and then was treated with ivy. The site was covered to avoid mechanical transfer and after 4 to 5 days was carefully freed from the incitant, or the whole isolated area was excised. When the animals were tested 6 to 8 days later, all were definitely hypersensitive, in varying degrees.

While this experiment in agreement with Simon's appeared to show

that interruption of epidermal continuity did not prevent general sensitization, we desired to attempt the experiment under more rigorous conditions. For this purpose a deep circular cut down to the muscles of the trunk was made on the back (or flank), thus isolating an island of skin, the edges, through contraction, being separated by a wide gap. By excision of additional strips of skin, depending upon the location, the gap was made uniformly wide (7 mm. or more).

TABLE II

Latency period preceding hypersensitivity to poison ivy. 0.05 cc. extract was applied to the sacral region and left for 4 days, covered with cellophane and linen patches; the residue then was removed with solvents and the site swabbed with KMnO_4 solution. Test applications on the back were made at the times noted after the ivy was placed on the skin, and concomitantly on additional animals as controls; the test doses were removed on the succeeding day. The table shows the 24 hour readings of the reactions to both dilutions, 1:20 and 1:40, separated by the slant line.

No.	Reactions to ivy extract applied at stated intervals following the primary administration					
	48 hrs.	3 days	4 days	5 days	6 days	7 days
21	0 / 0	f.tr. * / 0	tr. / 0	\pm / 0	++++ / ++	++++ / +++
22		0 / 0	0 / 0	++++ / +++	++++ / ++	+++ / +
23		0 / 0	0 / 0	++++ / +++	++++ / +++	
24		0 / 0	0 / 0	+++ / +	++++ / \pm	
25		tr. * / 0	tr. / 0	+++ / +	++++ / +++	++++ / +++
26			f.tr. * / 0	+++ / tr.	++++ / +++	++++ / +++
27			f.tr. * / 0	++++ / +	++++ / +++	++ \pm / +
28				+ / 0	+++ / \pm	+++ / +
29				++ / 0	+++ / +	+++ / ++
30				\pm / tr.	++++ / +++	++++ / ++ \pm
31					+++ / \pm	+++ / +
32					++++ / +	+++ / ++
33						+++ / +
34						++++ / ++++

* These reactions were less, or not greater than those exhibited by one or more of the 3 or 4 controls tested at the same time; on the average, about half of the controls showed trivial reactions (f.tr. or tr.) to the higher concentration.

With this technique the results were now different, sensitization being obtained in some cases but inconsistently and, with few exceptions, of low degree. In order to obtain more decisive results, several different procedures were tried. Finally definitely positive or negative sensitization effects across a dermal barrier were obtained almost regularly, depending upon whether or not the thin muscle layer underlying the cutis, the panniculus carnosus, was severed as well as the skin. A cut was made upon the flank circumscribing an area of skin; poison ivy

extract was applied in the center of this area, which then was carefully covered to prevent transfer of the active material to other parts of the integument. 4 or 5 days later the skin island, together with the protective coverings, was removed. By inspection it was easy to make sure at this time that no appreciable epithelial growth had taken place and that the edges of the epidermis were still wide apart; this macroscopic observation was confirmed by histological examination. Between the 10th and 14th days the animals were tested on various sites (see Figs. 1 to 4). The operations described were well tolerated, and within the 14 day period there was an actual gain in weight, averaging from 15 to 40 gm. with the different groups of animals.

The procedures will be described in some detail because they are of importance for obtaining clear cut results.

Flank Islands with Panniculus Carnosus Intact.—The position of the desired island was sketched with pencil on the clipped and shaved left flank, with the guinea pig in resting posture; after the animal under ether anesthesia had been fixed to the board on its side so as to maintain a right angle between trunk and extended hind limbs, without displacement of the loose flank skin, the outline was completed with the help of an ovoid stencil, 43 mm. by 37 mm. along the axes. (It might prove possible to make use of smaller islands in this type of experiment.) In an animal of about 550 gm. weight, the anterior midpoint of the line was, for instance, about 15 mm. posterior to the scapular angle. A second line was then drawn, to give a nearly rectangular figure, situated outside the first by 5 mm. at the dorsal and ventral midpoints, 6 to 7 mm. at the anterior and posterior midpoints, and separated by as much as 10 mm. along the diagonal diameters midway between dorso-ventral and antero-posterior diameters. (This technique assists in obtaining an island of circular shape, and a uniform width in the denuded ring.)

After the flank was sponged with alcohol, shallow incisions were made along both lines and carried cautiously down towards the panniculus carnosus, which appears as a grayish layer below the firm white connective tissue; the intervening skin was then carefully and sharply dissected from the panniculus, the procedure being facilitated by use of a binocular loupe. The field of dissection was kept moist with wet dressings, to reveal any residual bridges of dense connective tissue. Relatively little bleeding occurred. Upon contraction, the skin thus isolated becomes a nearly circular island of about 35 mm. diameter, surrounded by a ring-shaped defect about 8 mm. wide (Figs. 4, 5). When correctly done, which requires attention to detail, the operation will leave patent for the greater part the lymph vessels extending across the panniculus, as may be evidenced by intracutaneous injection into such islands of solutions of dyes (9) e.g., pontamine sky blue 6 B.

With the body protected against accidental contact, the ivy extract was applied to the center of the island in the manner described previously; then the cellophane and linen coverings were cemented to the island over the ivy material.

The wound was kept dry and clean by applying thymol iodide (Merck); this treatment was repeated several times, and thereafter once daily.

The claws of the hind feet were covered with boots of adhesive tape and, besides, the animals were prevented from disturbing the bandage by attachment of a cardboard collar or, preferably, by the following device: by means of a strip of cotton twill tape attached to an adhesive tape band around the right thigh and then affixed to a collar of iron wire covered with soft rubber tubing, worn around the neck, the leg was advanced toward the neck sufficiently to keep the trunk bent slightly sidewise, away from the flank island. This arrangement, maintained until subsequent excision of the islands, prevented narrowing of the defect and kept the cut surfaces from adhering, without otherwise interfering with the free movements of the animal. The isolated skin was surrounded by an even, circular moat, which remained dry (but for exceptional bleeding) and in which a crust appeared about the 3rd day. Frequent inspections were made, and occasionally blood clots or dried exudate were removed. (In the early experiments where cardboard collars were employed, free movements of the flank sometimes led to a sticking together of the opposing cut surfaces: whenever this occurred, the animals were withdrawn from the experiment.)

Flank Islands with the Panniculus Severed.—The location of the islands and the stencil used were the same as described above. The incision was extended downwards through the panniculus, whereupon the muscle contracted to give a gaping moat. Consequently, only narrow (3 to 4 mm.) strips of skin opposite the anterior and posterior margins of the island had to be removed in order to secure the same outer diameters of the ring (about 50 by 50 mm.) which obtained in the operations where the panniculus was left intact. Blood vessels were tied off when necessary. The isolated skin areas thus created measured about 29 mm. along the antero-posterior axis and 32 mm. dorso-ventrally (Fig. 3). (When dye is injected intracutaneously into such islands, it infiltrates the tissue and in some measure oozes out into the moat.) Application of ivy extract, protective coverings, and postoperative care followed the procedures in the section above; to be emphasized is the collar-to-leg method of restraint in order to maintain an even and dry moat despite the loose connective tissue between panniculus and trunk muscles. (By frequent application of thymol iodide, the wound dries satisfactorily, although a small area along the ventral surface may remain moist for a day or two.)

Excision of the Islands.—On the 4th day, exceptionally on the 5th, the hair near the island was clipped and, with the animal under deep ether anesthesia, the isolated skin with ivy material and coverings undisturbed was removed by dissection, usually sparing the panniculus carnosus. Vessels were clamped off and tied. Finally, the whole area was packed with thymol iodide and covered with sterile cotton cloth, which was cemented to the skin. In the case of the

islands where the skin muscle had been cut, there was edema by the 2nd or 3rd day, and during excision free fluid was present; the panniculus itself and the underlying tissues appeared thickened.

From the results of the two sorts of operations (Table III *a*), it is seen that of 25 guinea pigs in which an area of the skin had been isolated with severance of the skin muscle and the superficial lymph vessels, only two attained a marked degree of sensitivity and a few others gave some evidence of a slight sensitization; where the panniculus and the lymphatic trunks passing over its surface were spared, however, and this despite the need of care in the operation, all of the 25 animals became sensitive, almost regularly in high degree and comparable to the sensitization elicited by like treatment of normal animals (treated with group D, Table III *a*); one showed a low grade sensitivity and some a moderate hypersensitiveness. It will have been noted that a large dose was selected for the sensitizing application in these experiments (0.03 or 0.05 cc. of undiluted ivy extract); by this means the demonstration of the difference between the two operations was made more striking, but a large amount was not necessary, for smaller doses of ivy extract applied to the skin islands with intact skin muscle were sufficient to sensitize (Table III *b*).

It will be clear that the positive results can meet with no objections on technical grounds, particularly the possibility of contamination of the skin outside the island, a matter necessarily to be considered with so highly active a substance. Apart from the elaborate precautions taken, the two types of experiments were made in alternation and with identical technique (except for the difference in the cutting), and furnished convincing controls for one another.

It should be noted that a not inconsiderable percentage of positive sensitizations was obtained, in most cases, as mentioned, not of high degree, when "deep" cuts were made in other locations (principally on the back) than the one described; from some experiments with dye injections we are inclined to believe that this may be due, at least in part, to incomplete interruption of lymph passage.

Straus and Coca (2) as well as Schreus (4) have reported that in their experiments treatment of isolated parts of the skin resulted in local sensitization. Indeed that such a condition can occur seems evidenced by certain clinical experiences. Our somewhat limited attempts to achieve a local sensitivity restricted to a segregated area have so far not yielded a definitely positive result: we obtained either no sensitization at all, or a hypersensitiveness which included the

TABLE III a

Sensitization in relation to a skin defect encircling the site of application of poison ivy on the flank, (a) when the skin muscle is not involved in the operation, and (b) when the skin muscle is severed as well. The islands of treated skin were excised on the 4th (rarely the 5th) day, and between the 10th and 14th days the guinea pigs were tested for general hypersensitivity. With group A, 0.03 cc. ivy extract No. 1 was applied to the isolated skin, and the animals were finally tested with various dilutions thereof; on the remaining animals (groups B, C, D), 0.05 cc. ivy extract No. 2 was used for sensitization, and dilutions of the same extract were employed for the tests. Untreated animals were included as controls with each lot of guinea pigs tested. The reactions on two regions of the skin are shown in the table, the readings recorded being those made at 24 and 48 hours respectively.

Ivy applied to skin islands, panniculus carnosus intact										Ivy applied to skin islands, panniculus carnosus severed										Non-sensitized controls					
No.	Interval before test	days	Reaction on dorsum		Reaction on flank		Interval before test	days	Reaction on dorsum		Reaction on flank		No.	Reaction on dorsum		Reaction on flank									
			1:20	1:40	1:20	1:40			1:20	1:40	1:20	1:40		1:20	1:40	1:20	1:40								
Group A																									
35	13		+	+	+	+	13		0, 0	tr., 0	0, 0	47	f.tr., ±	0, 0	f.tr., tr.	0, 0									
36	13		+	+	+	+	13		0, 0	0, 0	0, 0	48	0, 0	0, 0	f.tr., ±	0, 0									
37	12		+	+	+	+	12		0, 0	0, 0	0, 0	49	0, 0	0, 0	0, 0	0, 0									
38	11		+	+	+	+	11		0, 0	0, 0	0, 0	50	0, 0	0, 0	tr., 0	0, 0									
39	11		+	+	+	+	11		f.tr., 0	0, 0	0, 0	51	0, 0	0, 0	0, tr.	0, 0									
40	10		+	+	+	+	10		0, 0	0, 0	0, 0	52	0, 0	0, 0	0, 0	0, 0									
Group B																									
53	14		+	+	+	+	13		±, ±	f.tr., tr.	0, 0	69	f.tr., f.tr.	0, 0	f.tr., ±	0, 0									
54	12		+	+	+	+	13		tr., tr.	0, 0	0, 0	70	0, 0	0, 0	0, f.tr.	0, 0									
55	12		+	+	+	+	12		+	+	+	71	±, tr.	0, 0	f.tr., f.tr.	0, 0									
56	12		+	+	+	+	12		f.tr., f.tr.	0, 0	f.tr., 0	72	f.tr., f.tr.	0, 0	±, tr.	0, 0									
57	11		+	+	+	+	11		0, 0	0, 0	0, 0														
58	11		+	+	+	+	11		±, f.tr.	f.tr., f.tr.	0, 0														
59	11		+	+	+	+	10		0, 0	f.tr., tr.	0, 0														
60	11		+	+	+	+	10		0, tr.	0, 0	0, 0														
Group C																									
73	14		+	+	+	+	14		±, tr.	f.tr., 0	0, 0	83	0, 0	0, 0	0, 0	0, 0									
74	14		+	+	+	+	14		±, ±	tr., 0	0, 0	84	tr., ±	f.tr., 0	0, 0	0, 0									
75	13		+	+	+	+	13		tr., f.tr.	0, 0	0, 0	85	0, 0	0, 0	0, 0	0, 0									
76	12		+	+	+	+	13		tr., ±	0, 0	0, 0														
77	12		+	+	+	+	13		f.tr., f.tr.	0, 0	0, 0														
Group D																									
86	12		+	+	+	+	12		tr., 0	0, 0	f.tr., 0	107	+, tr.	0, 0	tr., 0	0, 0									
87	12		+	+	+	+	12		f.tr., 0	0, 0	0, 0	108	0, 0	0, 0	0, f.tr.	0, 0									
88	12		+	+	+	+	12		+	+	+	109	f.tr., f.tr.	0, 0	0, f.tr.	0, 0									
89	12		+	+	+	+	11		tr., 0	0, 0	0, 0														
90	11		+	+	+	+	11		tr., 0	0, 0	0, 0														
91	10		+	+	+	+	10		tr., tr.	0, 0	0, 0														
Normal animals treated with ivy concurrently with group D and tested at the same time																									
Sensitization with 0.05 cc. of undiluted extract																									
98			+	+	+	+			+	+	+		1:15	1:30	1:15	1:30									
99			+	+	+	+			+	+	+														
100			+	+	+	+			+	+	+														
101			+	+	+	+			+	+	+														
102			+	+	+	+			+	+	+														
Sensitization with 0.05 cc. of 1:100 diluted extract																									
103			+	+	+	+			+	+	+														
104			+	+	+	+			+	+	+														
105			+	+	+	+			+	+	+														
106			+	+	+	+			+	+	+														

isolated area to which the substance (ivy extract, 2:4 dinitrochlorobenzene) had been applied, and the whole integument as well. Furthermore, when poison ivy was applied to a site of the intact skin and tests were made after a suitable interval, the intensity of the reaction on the treated site was if at all only slightly stronger than elsewhere (*cf.* 1).

A counterpart to the experiments described is the sensitization of a segregated skin area (made by a "deep" cut on the flank) when the active agent is administered on another part of the skin. Since under these conditions the island can be sensitive also, the spread through the circulation either of the allergen, probably transported in a changed state, or of antibodies is indicated. The reactions on the island were relatively weak, which may be due to alteration of the tissues. These experiments bear some relation to the "belt operation" of Simon (1).

TABLE III *b*

Same as Table III *a*, except that a smaller amount of ivy extract was applied to the skin islands (0.025 cc. of a 1:5 dilution in acetone of ivy extract No. 2). The animals were tested on the 10th or 11th days with dilutions of the same extract.

Ivy applied to skin islands, panniculus carnosus intact					Controls				
No.	Reactions on dorsum		Reactions on flank		No.	Reactions on dorsum		Reactions on flank	
	Dilutions		Dilutions			Dilutions		Dilutions	
	1:15	1:30	1:15	1:30		1:15	1:30	1:15	1:30
110	+++ , +++++	++ , +++	+, ++	±, +	116	tr., tr.	0, 0	tr., ±	0, tr.
111	+++ , +++++	+++ , +++++	+++ , +++	+, +	117	f.tr., 0	0, 0	0, 0	0, 0
112	+++ , +++	tr., tr.	+, +	±, ±	118	tr., tr.	0, 0	tr., ±	tr., f.tr.
113	+++ , +++++	++ , ++	++++ , +++	±, ±	119	±, tr.	0, 0	0, 0	0, 0
114	±, ++	tr., 0	±, ±	tr., ±	120	tr., tr.	tr., f.tr.	±, +	tr., 0
115	++++ , +++++	++++ , ++	+++ , +++	+++ , ++					

We next proceeded to investigate whether a cut through the skin and the superficial muscle would also interfere with anaphylactic sensitization to a common protein antigen if injected intracutaneously into the isolated area. Actually this was the case when small doses (0.0005 cc., 0.0002 cc.) of horse serum were used (Table IV); with larger amounts (0.0006 cc. or more) such an effect did not occur. This may show that there are other ways by which high molecular substances can be distributed from a skin site than the lymphatic vessels that were severed along with the skin muscle in our experiments; indeed this idea may be considered for the infrequent instances of positive sensitization with ivy from flank islands similarly isolated.

The positive results just mentioned with large doses of horse serum

may possibly be ascribed also to leakage of some of the protein into the defect from the cut lymphatic trunks. For it was seen that when diluted horse serum (0.0025 cc. contained in 0.3 cc.) was applied with

TABLE IV

Hindrance by a defect in skin and panniculus of anaphylactic sensitization to horse serum injected intracutaneously in small amounts. Skin islands on the flank with severance of the panniculus carnosus, as described for poison ivy, were prepared in guinea pigs weighing between 450 and 500 gm., and 0.1 cc. of diluted horse serum was injected intracutaneously into the center of the isolated skin or in the corresponding position on normal animals as controls. The islands, together with underlying panniculus, were excised on the 4th day. After 21 or 25 days the animals were injected intravenously with 0.3 cc. of horse serum contained in a volume of 1.0 or 1.5 cc. Figures in parentheses indicate change in temperature ($^{\circ}\text{C}.$).

Injection into skin islands, panniculus severed		Controls	
No.	Intravenous injection of horse serum	No.	Intravenous injection of horse serum
Sensitization with 0.0002 cc. serum			
121	No symptoms (+0.3)	127	Severe shock, recovered (-1.2)
122	Slight symptoms (-1.4)	128	† 9 min.*
123	" " (-1.0)	129	† 4 "
124	Severe shock, recovered (-2.8)	130	† 3 "
125	No symptoms (+0.5)		
126	" " (+0.9)		
Sensitization with 0.00005 cc. serum			
131	No symptoms (-0.4)	138	Moderate to severe (chronic type)
132	Very slight symptoms (+0.3)		(-3.5)
133	Slight to moderate symptoms (-0.5)	139	† 4 min.
134	No symptoms (+0.8)	140	Slight to moderate symptoms
135	" " (-0.5)		(-2.3)
136	" " (+1.0)	141	† 4 min.
137	Slight symptoms (-0.4)	142	† 3 "
		143	Severe symptoms (chronic type)
			(-3.5)
		144	† 3 min.

* The symbol † signifies death; the autopsy findings were typical in all cases.

a glass rod, slowly and with pauses, to the fresh wound surrounding flank islands that were made with severance of the panniculus carnosus, anaphylactic sensitivity frequently ensued, although not always with acute lethal shock (subsequent reinjection of horse serum as in

Table IV). It should be noted that the 0.0025 cc. amount used constitutes a rather large dose.

Similar exploratory experiments were then undertaken with another sensitizing substance of known structure, namely salvarsan (10), which induces skin sensitivity of a type different from the contact dermatitis of poison ivy. Here again, the barrier proved effective, for sensitization did not ensue when 0.15 mg. of salvarsan in 0.1 cc. was injected intracutaneously into flank islands (with the panniculus severed); the islands were excised on the 4th day, and tests for dermal sensitivity were made after 3 to 4 weeks by intracutaneous injection of a like amount.

DISCUSSION

From the experiments made in guinea pigs, which still leave problems for further investigation, the conclusion seems inescapable that continuity of the skin is not required for the development of general dermal sensitization to simple chemical compounds, since a broad defect in the entire thickness of the skin surrounding the area to which ivy extract is applied does not prevent hypersensitivity all over the skin. The significance of the question is clear from the literature reviewed. That there are differences as regards the route of distribution of the agent in various species would in our opinion seem quite improbable.

It is true that a "deep" cut down to the muscles of the trunk, made as described, inhibited sensitization almost regularly, and in this respect there is conformity with the results reported by Straus and Coca (2) for monkeys, and recently by Schreus (4) for guinea pigs. But upon a change in the experimental conditions the outcome was strikingly different, that is, when a strip of skin 5 to 10 mm. in width and comprising the whole thickness was excised in such manner as to spare the underlying skin muscle. The fact that the results differ depending upon the integrity of this muscle is apparently referable to the location of the lymph vessels draining the skin on the surface of the panniculus carnosus, with the consequence that cutting the muscle layer interrupts the lymphatics.² This can be demonstrated

² The anatomy of these vessels and the lymph nodes in the guinea pig are well described by Keller (11) whose drawings picture the superficial ramification of the lymph vessels. In this connection we may quote the conclusion of McMaster (12) that "every intradermal injection is truly intralymphatic."

by injecting intracutaneously into a freshly isolated island a solution of a colloidal dye, such as pontamine sky blue 6 B, for when the muscle is severed the dye chiefly is held locally, penetrating into the connective tissue, and to some extent oozes out from the severed lymph vessels; whereas, if the muscle is left intact, one sees the dye streaming across the moat through the lymphatics, and shortly the superficial and even the deeper regional lymph nodes are found upon dissection to be blue.³

Concerning the reasons for the interference with sensitization through severance of the lymph vessels, the most obvious is prevention or hindrance of transportation of the active material, if the latter is not such as to pass easily into the blood stream. In this respect it may be pointed out that sensitizing substances of simple constitution are probably in many instances not carried as such but rather in the form of some sort of conjugates (*cf.* 6), and indeed some of the compounds by their very instability (diazomethane (13), acyl chlorides (6)) must react rapidly when brought into contact with tissues; with poison ivy, as Simon (1) has reported, and this holds in our experience for other sensitizers of simple chemical constitution, direct introduction of the extract into the blood stream fails to induce skin hypersensitivity, from which fact he suggests that the active agent, if it is distributed by the blood, must have undergone some prior transformation.

In support of mechanical causes in preventing sensitization from skin islands isolated by a "deep" cut are experiments which show that anaphylactic sensitization by proteins, when one injects not too large amounts into such islands, also is definitely impaired (Table IV). Aside from hindrance to lymph flow, loss of some of the protein by leakage into the wound will occur, as with dye injected intracutaneously into the island.⁴ Whether much effective antigen is lost in this way is doubtful for dilute horse serum placed on the freshly made circular defect is in some measure taken up, since anaphylactic sensitization resulted from this treatment.

In the case of experimental ivy sensitization, there are in all probability factors additional to those which operate in the experiments

³ We are indebted for this technique to Drs. Austin L. Joyner and Philip D. McMaster.

⁴ In the experiments on poison ivy, with animals suitably restrained from activity, the base of the operated area remained dry and there was no indication of seepage from the cut edges.

with proteins, for with poison ivy, despite the use of an excess of ivy left in place on the isolated skin of such islands for several days, we find not merely a decrease in sensitization but in most cases nearly complete inhibition. That an essential difference exists in the mechanism is indeed known since proteins sensitize by any route while in order to induce skin sensitization towards simple compounds application of the incitant to or into the skin plays a special but probably not entirely mandatory,⁵ rôle. It is of interest that with a substance neither protein nor fat-soluble, salvarsan, sensitization was also seen to be entirely prevented following intradermal injection into an island, and here the resulting sensitization is not of the contact dermatitis type.

Why in the case studied of hypersensitiveness to poison ivy free lymph circulation is a necessity for the sensitization process we are not in a position to say. The existence of the highly developed lymph system of the skin (15, 9) may come into consideration,⁶ and also the altered state of the tissues in the skin island (*cf.* 8), as evidenced by a rather persistent edema; yet there well may be involved a disturbance of a special mechanism which is still unknown. A unique importance of the lymph glands themselves cannot be concluded without specific evidence, in view of other immunological knowledge, although the importance of these in the production of certain antibodies was demonstrated in experiments of McMaster *et al.* (18, 12).

To some extent the special conditions obtaining in the skin seem to play a part in sensitization with common protein antigens also. Thus Sulzberger (19) observed that the minimum amount of horse serum needed for anaphylactic sensitization was smaller when administered by the intradermal than the subcutaneous route, a result which we were able to verify in more extensive tests, although the ratio of the minimum doses varied from one experiment to another.

The production of a local sensitivity by application of ivy to an island has so far not succeeded, and the question may still be open

⁵ Experiments in progress appear to show that a dermal sensitization may be induced by intraperitoneal injection with the aid of adjuvants, *e.g.* with picryl chloride (*vide* 14) and killed tubercle bacilli.

⁶ As regards peculiarities of the immunological rôle of the skin tissues, see (16, 17).

whether the process of sensitization can be completed in or by the dermal tissues alone; on the other hand, a part of the skin isolated with interruption of the lymphatics can take part in the general sensitivity produced upon treatment of the skin outside, and this is hardly to be explained otherwise than by the intermediacy of the blood stream.

SUMMARY

Experiments are described on the latency period in sensitization to poison ivy and on the time necessary for the agent to remain in contact with the skin. The chief matter of investigation concerned the manner in which the whole skin becomes sensitive following treatment at a particular site, and especially whether this is effected by way of the epidermis.

Two methods were used to interrupt the continuity of the skin, one by cutting through both skin and the underlying thin muscular layer, the other by removing a strip of skin so as to spare the skin muscle. These procedures led to different results when poison ivy extract was applied to the areas thus isolated. In the first case, sensitization was mostly prevented, whereas with the second method generalized hypersensitiveness occurred almost uniformly.

An explanation is to be found in the severance of the lymph vessels lying on the surface of the muscular layer, pointing to the necessity of a free lymph passage. On the other hand the experiments prove that general sensitization is not dependent upon maintaining the integrity of the skin around a treated area.

An inhibition of sensitization by incisions extending through the panniculus carnosus was seen to some extent in anaphylactic sensitization with protein antigens, namely when sufficiently small amounts were employed.

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EXPLANATION OF PLATE 42

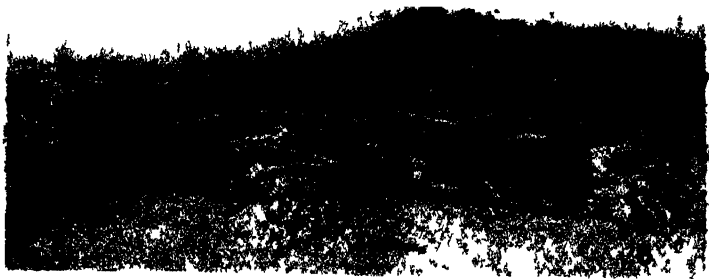
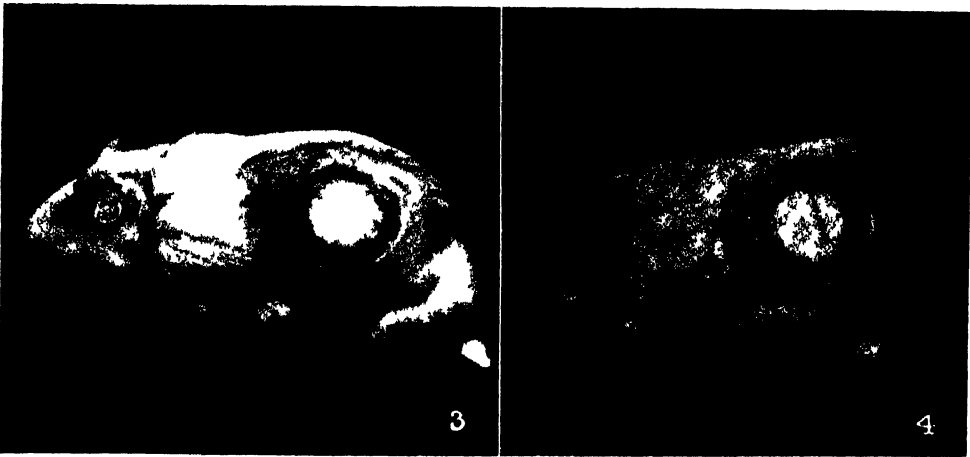
FIG. 1. Test for hypersensitiveness on the 14th day after operation (and ivy treatment) as shown in Fig. 3, with two different concentrations of ivy extract.

FIG. 2. Similar test after the operation as shown in Fig. 4.

FIG. 3. Isolated area of skin on the flank, with severance of the panniculus carnosus, after applying poison ivy and dressing.

FIG. 4. Skin island as above, but with panniculus carnosus spared.

FIG. 5. Section across the marginal part of the skin defect made as in Fig. 4. The skin muscle is intact and is covered on the larger (central) part with only a thin layer of loose connective tissue. $\times 8$.



Photographed by Joseph B Haulenbeek

(Landsteiner and Chase Sensitization with chemical compounds VI)

SPONTANEOUSLY ACQUIRED TUBERCULOSIS IN RHESUS MONKEYS

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In earlier publications we have reported our studies (1, 2) on the colonial morphology and virulence of tubercle bacilli isolated from various sources. Similar studies have been continued since these reports appeared, and among the many strains of tubercle bacilli isolated prior to January, 1938, 4 were from rhesus monkeys. The animals from which these 4 strains were isolated were in the colony of Dr. P. K. Olitsky to whom we are indebted for this and a part of the later material. Each of the four animals had acquired the disease spontaneously. When the pathogenic properties of these 4 strains were studied it was found that 3 of them were highly virulent for rabbits, as well as for guinea pigs. They were therefore of bovine type.

This high incidence of bovine type infection among the strains first isolated raised two questions: (a) whether this high incidence of bovine type infection is constant and, if so, how can it be explained when the incidence of bovine tuberculosis among cattle and among humans is so low in America; and (b) whether monkeys contract tuberculosis while under experimental observation, or prior to experimental use. Investigations were begun in January, 1938 to elucidate these two points and this report includes the observations made to date.¹

MATERIALS AND METHODS

Whenever a monkey was discovered at necropsy to have had tuberculosis, a survey was made to ascertain, if possible, whether the primary lesion was situated in the respiratory or gastrointestinal system. Notes

¹ We wish here to acknowledge and express gratitude for the generosity of Doctors P. K. Olitsky and A. B. Sabin of this Institute and of Doctors J. H. Bauer and L. T. Coggeshall of the International Health Division of the Rockefeller Foundation, who have made available to us the tissues of infected monkeys from their respective colonies, together with records of the infected animals.

were made on the distribution and extent of lesions, and blocks of infected viscera were saved for the preparation of histological sections. A portion of the most heavily infected viscus was reserved for animal inoculations and for making cultures.

Animal inoculations: A block of tissue, usually spleen or lung from the tuberculous monkey, was minced with sterile scissors, then ground in a mortar with sterile sand and sterile saline solution to make a heavy suspension, usually of about 20 per cent strength. Five cc. of this suspension were then centrifuged at very low speed for five minutes to throw down the largest particles, and the turbid supernatant was used for subcutaneous inoculation of guinea pigs. Each animal received about 2.0 cc. of the centrifuged suspension subcutaneously in the right groin. The suspensions were usually contaminated with other bacteria than tubercle bacilli, but none of the guinea pigs died from extraneous cause.

Cultures: To the remainder of the crude suspension of tissue from each diseased monkey was added an equal volume of 6 per cent (by weight) sulphuric acid and a few drops of bromthymol blue indicator. The flask containing this mixture was agitated and allowed to stand at room temperature. After thirty minutes or one hour the contents of the flask were brought to neutrality by adding 3 per cent sodium hydroxide. Several tubes of glycerolated egg-yolk medium (3) adjusted to pH 6.8 were seeded with about 0.25 cc. each of the neutral tissue suspension. The tubes were sealed with melted paraffin and incubated at 37°C.

In several instances some of the cultures so prepared were contaminated, either with postmortem invaders, or because necropsies were not done under aseptic conditions. For this reason each strain was also isolated after passage through a guinea pig. At a time after inoculation when one could be certain that the disease was established in the guinea pig (thirty to sixty days following inoculation) each was sacrificed and the spleen removed under aseptic conditions. About half the spleen was used to make cultures in the manner described above, except that the guinea pig tissues were exposed to sulphuric acid for only fifteen or twenty minutes.

When each strain had grown well, either in primary culture or after isolation from a guinea pig, it was transferred routinely on Corper's medium (3). Studies of the colony morphology were made on both the primary cultures and the stock cultures.

Typing of strains according to virulence: Four of the strains isolated in this study were typed by means of virulence tests. Two normal rabbits and two normal guinea pigs were each inoculated with 0.1 mg. of a stock culture of the strain to be tested. Rabbits were inoculated intravenously, guinea pigs subcutaneously. One rabbit and one guinea pig were sacrificed thirty days following inoculation, and all survivors at sixty days. Careful records were kept of the extent of lesions observed at autopsy, and microscopical surveys were also made to determine the extent of lesions. In this manner an approximation of the virulence of the organisms could be gained from the guinea pigs; and the progression or regression of lesions in rabbits indicated the type of the organisms (bovine if lesions progressed after thirty days, human type if regression occurred).

Typing of strains according to colonial morphology: We had observed previously (4) that the usual morphology of bovine tubercle bacilli grown on egg-yolk medium adjusted to pH 6.8 is smooth and glistening. When growth is massive the hemispherical contour is lost but the glistening, moist appearance remains and the margins of the growth are sharp and regular. Human type strains cultivated under identical conditions form colonies which are often quite round at the base, but dry and finely granular in consistency, and usually with elevated centres. Massive growths are also dry and finely granular, and from the edge of the growth a thin veil projects. Moreover, growth of human strains is, as a rule, more vigorous and profuse.

Using the criteria described above, we examined as unknowns a large number of subcultures of strains of known type and found that the type of organisms could be ascertained with a very considerable degree of certainty. Accordingly the primary cultures isolated in this study, either directly from monkeys or after passage through a guinea pig, together with the stock subcultures of each strain, were examined with a magnifying glass and type determinations were made. In each instance the type determination on the primary cultures agreed with similar determinations on the stock cultures. Such studies were made after three or four weeks' incubation as it was previously found (4) that studies made later were subject to changes and were less reliable.

VIRULENCE OF THE STRAINS ISOLATED FROM MONKEYS

The virulence of three strains has not been determined except as they induced progressive disease in monkeys. Each of the 4 strains which

were inoculated into rabbits and guinea pigs were fully virulent for guinea pigs, and 3 of them were fully virulent for rabbits also. Eleven additional strains were each inoculated into two guinea pigs. One strain (6262, table 1) proved to be of substandard virulence, while the remaining 10 strains were fully pathogenic for cavies. The approximate virulence of the 15 strains so studied is shown in table 1.

In the case of strain 6127 the tissues of the monkey from which the strain was isolated were heavily laden with acid-fast bacteria. The organisms were present in such numbers that they could be seen in sections with the low power lens ($\times 150$). The lesions in this monkey were massively caseous. One of the two guinea pigs inoculated with the suspension of spleen from this monkey was sacrificed after thirty days and exhibited rather extensive pulmonary, hepatic and splenic lesions

TABLE 1
Relative virulence of 15 strains of tubercle bacilli isolated from monkeys

	STRAIN NUMBER														
	3103	4540	4861	4892	6127	6187	6240	6241	6242	6258	6259	6262	6263	6293	6373
Virulence in guinea pigs.....	4+	4+	4+	4+	4+*	4+	4+	4+	4+	4+	4+	2+	4+	4+	4+
Virulence in rabbits.....	±	4+	4+	4+	†	†	†	†	†	†	†	†	†	†	†

4+ indicates highly pathogenic, 2+ indicates moderately pathogenic, ± indicates very slightly pathogenic (no progressive disease).

* See remarks in text regarding pathogenic properties of this strain.

† Indicates test not done.

with extensive caseation and numerous acid-fast bacilli in the lesions. The second guinea pig succumbed on the fifty-fourth day following inoculation and exhibited extensive lesions in spleen, lungs, liver and lymph nodes. The lesions seen microscopically were not epithelioid cell tubercles but were small aggregations of free and phagocytosed neutrophils and phagocytic mononuclears, each bearing many acid-fast bacilli. In the lungs these collections of cells were intraalveolar, and the bordering alveolar septa showed no change other than slight vascular dilatation. But the cells within the alveoli showed marked degenerative change, and, as in the monkey from which the strain originated, the acid-fast organisms were so numerous they could be seen at low magnification. These findings are detailed because of the extraordinary pathological picture induced by strain 6127.

TYPES OF MYCOBACTERIA RECOVERED FROM MONKEYS

The type of 4 strains was determined by inoculation of rabbits and guinea pigs. Fourteen additional strains were isolated from as many monkeys and were typed according to their colonial morphology. Regarding the 4 strains typed by virulence, the result is quite certain. In the case of the strains typed by colonial morphology, the result is presumably, though not certainly, correct in every instance. Table 2

TABLE 2

Cultural characteristics and type of each of 18 strains of tubercle bacilli isolated from 18 rhesus monkeys

STRAIN FROM RHESUS NUMBER	VIGOR OF GROWTH	PREDOMINANT COLONY FORM	MANNER OF TYPING	TYPE OF STRAIN
3103*	Excellent	FS†	Virulence	Human
4540	Fair	S	Virulence	Bovine
4861	Fair	S	Virulence	Bovine
4892	Fair	S	Virulence	Bovine
6127	Poor	S	Cultural characteristics	Bovine
6187	Excellent	FS	Cultural characteristics	Human
6240	Fair	FS	Cultural characteristics	Human
6241	Fair	FS	Cultural characteristics	Human
6242	Fair	S	Cultural characteristics	Bovine
6258	Poor	S	Cultural characteristics	Bovine
6259	Good	FS	Cultural characteristics	Human
6262	Good	FS + R	Cultural characteristics	Human
6263	Good	FS	Cultural characteristics	Human
6293	Poor	S	Cultural characteristics	Bovine
6373	Poor	S	Cultural characteristics	Bovine
6422	Excellent	FS + R	Cultural characteristics	Human
6423	Good	FS + R	Cultural characteristics	Human
6424	Good	S	Cultural characteristics	Bovine

* These are serial numbers used over a period of many years.

† FS indicates flat spreading granular colonies, S indicates smooth, and R indicates coarsely granular rough colonies.

shows the type of the 18 strains, together with their cultural characteristics. The first 4 strains included in table 2 were those typed by virulence tests.

Table 2 shows that between the bovine and human type strains there were differences in both colony form and growth energy.

This result shows that the incidence of bovine type infection among the 18 rhesus monkeys was 50 per cent. While this figure cannot be re-

garded as rigidly accurate since the type of each strain was not determined by inoculation of animals, the incidence is certainly much higher than the incidence of bovine type infections among human beings in America. Moreover, the high incidence of bovine infection could not be accounted for on the basis of the diet supplied to the animals after arrival in the United States, since herd-testing and pasteurization of the milk supply is practiced. The obvious assumption was, therefore, that at least a part of the monkeys contracted tuberculosis before arrival in this country. Subsequent evidence will be offered to substantiate this assumption.

TIME WHEN DISEASE WAS ACQUIRED BY MONKEYS

Obviously it was not possible to ascertain in every instance whether monkeys contracted tuberculosis in the laboratories or elsewhere. But in four animals from which tubercle bacilli were isolated we had available the complete histories from the date of their arrival by steamship. These animals, 6242, 6258, 6262 and 6263 (table 2), arrived by boat at New York on December 28, 1937. They were received by the dealer, transported by him to this Institute on January 4, 1938, and were included in a group of forty-five monkeys received by Dr. P. K. Olitsky and Dr. A. B. Sabin. The discovery of tuberculosis in three of them (6258, 6262 and 6263) was incidental to the necropsy following other experimental procedures, that is, the animals did not die as a result of tuberculosis. In rhesus 6258 the pulmonary lesions had already advanced to cavitation, and in 6263 there was a large, firm but fluctuant tuberculous abscess at the ileocaecal junction. These lesions were certainly not fresh ones, yet rhesus 6258 and 6263 had arrived at New York only thirty-four and thirty-seven days prior to the time the lesions were found. Rhesus 6262, sacrificed thirty-six days after arrival, exhibited lesions in the gut, mesentery, omentum, spleen, liver and lungs. The oldest lesions were in the liver and lungs. More convincing still is the record of rhesus 6242. This animal was kept on a normal diet and subjected to no experimental procedure, yet died on January 25, 1938, just twenty-eight days after arrival in the United States. Emaciation was marked. At autopsy two chronic tuberculous lesions were found in the lung. Opposite one of these there was a dense fibrous adhesion to the parietal pleura. One lesion was caseous; the other had advanced to the fluid state. Tubercle bacilli were numerous in the lesions.

As further evidence that at least some of the animals were infected with tuberculosis prior to entry to America is the fact that in six of twelve instances, in which the primary lesions could be ascertained,³ this was in the gastrointestinal tract. This would seem to point to a contaminated food supply. Monkeys in this Institute are given a diet consisting of pasteurized milk, bread, bananas, oranges, peanuts and carrots. Moreover, the handlers are healthy and apparently free of tuberculosis, so that it would seem probable that the infections were acquired elsewhere.

These results make it seem highly probable that a certain percentage of laboratory monkeys which have tuberculosis acquire the disease before arrival in America. In transit or in the animal quarters of the laboratories they may infect other animals by coughing or otherwise; but in a small survey it appears that about 9 per cent of animals of a given lot (4 of 45) were tuberculous on arrival.

DISCUSSION

One of the primary requisites for experimental investigations is dependable animal stock. The existence of tuberculosis may significantly influence the course of events in animals being used for dietary studies, or investigations of other diseases, or even in physiological experimentation. The rhesus monkey is essential for investigation in certain fields. This animal has been known to be highly susceptible to tuberculosis, yet is said to be rarely if ever infected with tuberculosis when in the wild state. The results we have obtained indicate, however, that a considerable number of monkeys may become infected with tuberculosis before they reach America. Obviously a useful purpose would be served if tuberculosis-free stock were available for experimental use.

However important a tuberculosis-free supply of monkeys would be to medical research, it would seem even more important that the potential public health hazard of the tuberculous monkey be studied. Our observations indicate that pulmonary lesions in monkeys frequently cavitate; they must expel many bacteria with their coughing. It is reasonable to assume that if monkeys imported for research purposes

³ In all of the monkeys of this series lesions were present in lungs as well as in abdominal viscera. It was not possible in all cases to discover the primary lesion. But when there was a large, old lesion in the gastrointestinal system and only miliary lesions in the lung, the lesion was considered to have originated in the former.

are tuberculous, so also are monkeys imported for exhibition in our large public zoological gardens. Then, unless adequate precautions are taken, tuberculous monkeys are placed on exhibition in overcrowded cities and visited by throngs which include many undernourished children—fertile soil for continued proliferation of mycobacteria. Indeed it may well be that the rhesus monkey is the source of the small residuum of bovine tuberculosis in our cities, and of a certain additional percentage of human type infections. Of these facts some pathologists in our zoos are well aware. Fox (5) discusses at length the existence of tuberculosis in monkeys, and describes methods for its control which have been successful in the Philadelphia Zoo. Schroeder (6) has recently written on the subject. He pointed to the fact that primates in the wild are free of tuberculosis, that heavy exposure, beginning immediately with capture, continues during transit and while animals are retained by dealers. He emphasized the hazard to the monkey of uncontrolled exhibition in public places but failed to point out the hazard of the monkey to man, although he stated that in primates in captivity the mortality rate from tuberculosis is two hundred times that in man. Therefore it would seem that rigorous steps are justified in order to prevent the importation of tuberculous monkeys. Health measures instituted at the source of origin and regulations governing the shipment and handling of monkeys by dealers might accomplish the desired effect.

SUMMARY

Eighteen strains of tubercle bacilli were isolated from as many cases of spontaneously acquired tuberculosis in rhesus monkeys. In about half the monkeys the primary lesion was apparently in the gastrointestinal tract. Fifty per cent of the strains were of bovine type. Evidence was gathered which indicates that a portion, at least, of the animals were tuberculous when imported.

The importation of tuberculous monkeys for zoological exhibition may constitute a public health hazard.

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THE EFFECTS OF ULTRAVIOLET RADIATION ON TUBERCLE BACILLI

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Among workers studying tuberculosis the interest in ultraviolet radiation has centered chiefly on its clinical application. However, it was early recognized that the sun's rays have lethal effects on tubercle bacilli, and subsequently shown that this action was chiefly due to rays in the ultraviolet region. A review of the earlier studies on the clinical use of ultraviolet radiations, and on the effects of these radiations on tubercle bacilli, was published in 1921 by Mayer (1). In 1924 Mayer and Dworski (2) employed rather weak but not well standardized suspensions of tubercle bacilli and found that the organisms were killed by as little as three minutes' exposure at a distance of 5 inches, by the radiation emitted from a quartz mercury vapor lamp. Howze (3) found that tubercle bacilli were killed in five minutes by the radiation of a mercury vapor lamp, but he did not standardize the suspensions and his experiments cannot be regarded as quantitative. Eidinow (4) observed that saline suspensions containing 1 mg. of tubercle bacilli per cc. were rendered nonvirulent by ten or fifteen minutes' exposure to the mercury vapor lamp and believed the rays shorter than 3,300 Å to be the most effective. A year later Mayer and Dworski (5) reported that suspensions containing 2,750,000 tubercle bacilli per cc., exposed at a distance of 2.5 cm. from the window of the lamp, were reduced in pathogenicity in two or three minutes and were made nonvirulent by four minutes' exposure.

Our interest in this subject was stimulated by the work of Stanley (6), Hodes, Lavin and Webster (7), and Kidd (8). Stanley (6) showed that tobacco-mosaic virus protein could be rendered avirulent by ultraviolet radiation, while the immunological activity was retained. Kidd (8) likewise found that the infectivity of the Shope papilloma virus could be abolished by means of ultraviolet radiation without loss of complement binding capacity. Hodes, Lavin and Webster (7) made

different application of the same principles; they found that the infectivity of rabies virus could be eliminated with ultraviolet radiation, and that if the exposure was quantitatively proper the inactivated virus was still able to induce immunity to rabies in mice.

Lavin and Stanley (9) found that the tobacco-mosaic virus protein had its maximum absorption at about 2,650 Å. Burger (10) found that radiation in this range had much greater bactericidal action than did longer wave lengths; he also found that bacteria inactivated with ultraviolet rays made superior vaccines (11). Gates (12) later showed that the maximum absorption of *B. coli* was in the same approximate range and that similar radiation was highly effective in the inactivation of both *Staphylococcus aureus* and its specific bacteriophage (13). Furthermore Spiegel-Adolf and Seibert (14, 15) observed that various tuberculin preparations had absorption maxima at 2,650 Å. A lamp was commercially available which had a maximum energy output in this approximate region. It was, therefore, decided to use this lamp to study the effect of its radiation on the immunizing potency of tubercle bacilli. Simultaneously, studies were made of the effects of the radiation on the viability and virulence of mycobacteria.

MATERIALS AND METHODS

Ultraviolet radiation: The source of radiation employed was a mercury vapor resonance lamp (manufactured by Hanovia Chemical Co., Newark, New Jersey). Approximately 90 per cent of the energy was emitted as radiation of 2,537 Å. Suspensions of tubercle bacilli contained in quartz flasks were exposed to the lamp while being agitated in a mechanical shaker so arranged that the flask was at a constant distance (15 cm.) from the source of radiation. During exposure the quartz flask was sealed with a close fitting gum-rubber cap. The physical set-up is described in detail by Hodes, Lavin and Webster (16).

Intervals of exposure were timed with a stop-watch. At the end of an interval the lamp was screened, the gum-rubber cap was removed from the quartz flask, and the sample of irradiated suspension was removed with a sterile pipette.

Bacteria: Two strains of tubercle bacilli were used in the experiments: a highly virulent line of the well known human strain H37, and a second human type strain designated Lockett, isolated from the cerebrospinal fluid of a patient with tuberculous meningitis (17). The organisms were grown on glycerolated egg-yolk medium adjusted to pH 6.8 (18). Vigor-

ously growing cultures, about three weeks old, were employed. Suspensions were prepared by grinding a weighed quantity of organisms, freshly removed from the tube, in a sterile porcelaine mortar, with dropwise addition of sterile physiological saline solution in sufficient quantity so that 1 cc. of suspension contained 1 mg. of bacteria. Appropriate decimal dilutions were made from such suspensions to contain the desired quantity of bacteria. Suspensions to be irradiated were at once transferred to the sterile quartz flasks, and suspensions used for inoculation were employed promptly to minimize the sedimentation or agglutination of the organisms.

Animals: Albino guinea pigs, bred in this Institute from stock free of epizootic streptococcal infection, were used in the experiments. Both males and females were used, but animals of different sexes were caged separately. In experiments where groups of animals were to be compared for longevity following inoculation, the groups were arranged either to contain animals of the same sex, or so that the distribution of individuals of different sex was the same in control and test groups. Also, groups to be so compared were comprised of animals of similar individual weights. For the most part the guinea pigs weighed about 400 g. Not more than 4 were caged together.

Inoculations: All inoculations, whether for the purpose of testing the virulence of irradiated organisms, or to test the immunizing potency of vaccine, were made by the intracerebral route. Anaesthesia was induced with ether or by intraperitoneal injection of 0.5 per cent solution of Seconal¹ in saline. The dose of Seconal was 20 mg. per kg. Intracerebral inoculations were done by the method previously described (19).

Vaccinations: Irradiated suspensions containing 1.0 mg. of bacteria per cc. were injected subcutaneously along the right side. Injections were made daily for five days, the daily dose being 0.3 mg. in 0.3 cc. In one experiment test inoculations were done eleven days following the last dose of vaccine. In the other experiment the animals were inoculated fifteen days following the last prophylactic injection.

Tests of viability of irradiated suspensions: These were made by seeding tubes of the glycerolated egg-yolk medium with 0.2 cc. each of suspension. In two experiments tubes were seeded in duplicate; in other experiments 5 tubes were seeded with each irradiated sample.

¹ Sodium Propyl-methyl-carbinyl Allyl Barbiturate, Lilly, generously supplied by Eli Lilly and Company, through the courtesy of Dr. G. F. Kempf, Indianapolis City Hospital.

Control cultures were also made of the nonirradiated suspensions. The tubes were sealed with melted paraffin and incubated at 37°C. They were examined weekly with a hand lens and records were made of the time when growth appeared, of the number of tubes showing growth and of the approximate number of colonies per tube. Final recordings of failure of growth were made only after eight or more weeks' incubation.

Tests of virulence of irradiated suspensions: These were made by inoculating irradiated and control (nonirradiated) suspensions intracerebrally. Two normal animals were used to test each suspension. The dose of organisms was 0.01 mg. or larger. (This dose of virulent organisms introduced intracerebrally usually causes death in about three weeks.) Organisms were considered nonvirulent only when both animals inoculated remained symptom free for six weeks or longer.

Tests of immunization: Two varieties of such tests were made. First, animals which survived the intracerebral injection of a single dose of irradiated organisms (0.01 mg.) and remained well for six or more weeks were reinoculated intracerebrally, together with comparable normal controls, with a small dose of virulent organisms. Second, groups of guinea pigs which had been vaccinated subcutaneously with irradiated organisms, together with normal controls of comparable age, sex and weight, were inoculated intracerebrally with 0.000,01 mg. each of the homologous virulent strain of organisms. In such experiments the results were measured by longevity following virulent inoculation, and the significance of the result was determined by standard methods (20).

EFFECT OF IRRADIATION ON VIABILITY OF TUBERCLE BACILLI

Four separate suspensions of three different densities of human tubercle bacilli, strain H37, were irradiated with the mercury resonance lamp and samples were withdrawn at the intervals shown in table 1. Two to 5 tubes of glycerolated egg-yolk medium were seeded with each sample, sealed and incubated. The final result of the tests is shown in table 1.

It will be noted from the data that, with the least dense suspension (0.05 mg. per cc.), no growth was obtained in any of 60 tubes seeded with irradiated organisms, but the nonirradiated organisms grew massively. The organisms were apparently rendered nonviable by as little as one minute's exposure to the lamp. The suspension of inter-

mediate density (0.1 mg. per cc.) contained a very few viable bacteria after two minutes' exposure to the radiation. One of 2 tubes seeded with this sample showed a single colony; the duplicate tube was negative. However, with a suspension containing 1.0 mg. of organisms per cc., there were still viable organisms after nine minutes' irradiation, while cultures of the ten-minute sample remained negative. This latter confirmed the observation made many years ago by Henri-Cernovodeanu, Henri and Baroni (21). The fact remains unexplained that the three-

TABLE 1

Growth of human tubercle bacilli strain H37 after irradiation for various lengths of time

IRRADIATION <i>minutes</i>	DENSITY OF SUSPENSIONS EXPRESSED AS MG. PER CC.			
	0.05†	0.1	1.0	1.0
Control 0	5/5*	2/2	2/2	5/5
0.5		1/2	2/2	
1.0	0/5	0/2		5/5
1.5		0/2		
2.0	0/5	1/2		5/5
2.5			2/2	
3.0	0/5			0/5
4.0	0/5			1/5
5.0	0/5			2/5
6.0	0/5			5/5
7.0	0/5			5/5
8.0	0/5			2/5
9.0	0/5			5/5
10.0	0/5			0/5
12.5	0/5			
15.0	0/5			

* Numerator = number of tubes showing growth. Denominator = number of tubes planted.

† This suspension was cleared of clumps by light centrifugation, then brought to desired density by comparison of turbidity with a standard suspension.

minute sample yielded no growth, and that there were negative tubes from the four- and five-minute samples of this suspension, while all tubes seeded from the six- and seven-minute samples showed growth. It must be remarked, however, that none of the irradiated samples yielded growth equal in quantity to the control nonirradiated samples; and in general it was true that samples irradiated successively longer showed progressively fewer colonies.

Smears of the irradiated bacteria were prepared and stained by the

Cooper method. All the samples shown in table 1 retained their acid-fastness and could not be differentiated in the smears from the control nonirradiated organisms. This observation is contrary to that of Cernovodeanu and Henri (22).

This result indicated that the radiation emitted by the lamp used was capable of quickly rendering tubercle bacilli nonviable when the suspensions were relatively weak, and showed that with more dense suspensions the time required to render the organisms nonviable was considerably prolonged. Furthermore, very short exposure to radiation was effective in reducing the number of viable organisms, but longer exposure was necessary to kill them all.

EFFECT OF IRRADIATION ON VIRULENCE

Three experiments were done to test the virulence of organisms exposed to radiation for varying lengths of time. The nonirradiated and irradiated samples of each suspension were inoculated intracerebrally, each sample into 2 normal albino guinea pigs. Relatively large doses were injected (not less than 0.01 mg.) in order to be able to detect the presence of small numbers of virulent organisms which might survive the irradiation. The H37 strain used in two of these experiments was fully virulent, the Lockett strain slightly less so. From prior experience it was known that 0.01 mg. of fully virulent organisms ordinarily causes death within about three weeks after intracerebral inoculation; and 0.000,001 mg. usually causes death in less than six weeks (19). The experiments were therefore not terminated until six or more weeks after inoculation. Table 2 shows the results of the experiment.

The results shown in table 2 indicated that the Lockett organisms in suspension containing 0.1 mg. per cc. were nonvirulent after two minutes' exposure to the lamp. These animals were observed for sixty-seven days and none showed any ill effects from the inoculations; the control animals inoculated with nonirradiated organisms both succumbed. The middle vertical column in table 2 shows that a similar suspension of the H37 strain was rendered less virulent (the animals living longer than those inoculated with the nonirradiated suspension) by as little as fifteen seconds' exposure to the radiation, and nonvirulent by thirty seconds' exposure. This was the same experiment as that in which a single colony was obtained in 1 of 2 tubes seeded with suspension irradiated two minutes (table 1). The result, therefore, indicated that although viable organisms were present they had either lost their viru-

lence or else were present in such small numbers as to allow survival without evidence of illness for forty-two days after inoculation. The experiment in the last column to the right in table 2 showed that in a heavier suspension of the H37 strain virulent organisms remained after two and one-half minutes' irradiation. This confirmed the result obtained by cultural methods (table 1) and showed that greater exposure to radiation is required for dense suspensions in order to reduce the virulence of the organisms or the number which are viable.

TABLE 2

Virulence of human tubercle bacilli after irradiation, as determined by survival or death following intracerebral inoculation

IRRADIATION	LOCKETT STRAIN 0.01 MG.*	H37 STRAIN 0.01 MG.	H37 STRAIN 0.1 MG.
<i>minutes</i>			
0.25		D 36† D 31	
0.5		S	D 27 D 44
0.75		S	
1.0		S	
1.25		S	
1.5		S	
1.75		S	
2.0	S	S	
2.5			D 29 D 36
4.0		S	
6.0	S		
10.0	S		
14	S		
Control } 0 }	D 30 D 31	D 23 D 24	

* This dose was inoculated in 0.1 cc. saline. The suspension irradiated therefore contained ten times this quantity per 1 cc.

† D indicates death on day shown. S indicates survival of both animals inoculated with a sample.

In summary of the tests of virulence we may say that an unknown number of organisms remained viable and virulent after two and one-half minutes' irradiation of a suspension containing 1.0 mg. of organisms per cc. When the suspension contained only 0.1 mg. of organisms per cc., fifteen seconds' exposure was adequate to appreciably reduce the virulence of the organisms (either by attenuation or by killing off a portion of them) and thirty seconds of exposure to the lamp rendered the organisms nonvirulent.

TESTS OF IMMUNITY AFTER INJECTION OF IRRADIATED ORGANISMS

Each of the animals (table 2) which survived the intracerebral inoculation of 0.01 mg. of either the irradiated Lockett or the irradiated H37 organisms was reinoculated intracerebrally with 0.000,01 mg. of the homologous virulent organisms to ascertain whether the small quantity of organisms made nonvirulent by irradiation had induced measurable immunity. Normal control guinea pigs were inoculated at the same time with the same dose of organisms. All these animals succumbed to tuberculosis, and none of those first inoculated with irradiated organisms were more resistant to the test inoculation than the normal controls. This result indicated that no appreciable immunity is induced by a single intracerebral injection of 0.01 mg. of irradiated tubercle bacilli.

Two additional experiments were done to determine whether enhanced resistance could be induced by subcutaneous injection of larger numbers of irradiated organisms. In both experiments the suspension irradiated contained 1.0 mg. per cc. of the strain H37. In the first experiment two irradiated samples were used as vaccine: one had been exposed to the lamp thirty seconds and the other two and one-half minutes. Both these specimens were found to contain a residuum of viable, virulent organisms (tables 1 and 2). In the second experiment two samples of vaccine were also used. One had been exposed to radiation for five minutes, the other for ten minutes. The five-minute sample was found to contain a few viable organisms (table 1) the virulence of which was not tested. Bacteriological tests indicated that the sample irradiated ten minutes contained no viable organisms. Each of the four irradiated samples of mycobacteria was injected subcutaneously daily for five days in each of a group of 5 or 8 normal guinea pigs. The daily dose for each animal was 0.3 mg. Eleven or fifteen days following the final injections of vaccine, these guinea pigs and comparable groups of non-vaccinated control animals were inoculated intracerebrally with 0.000,01 mg. of the homologous virulent organisms to test their resistance. None of the animals was submitted to other experimental procedure. The criterion of resistance was the length of survival following test inoculation. The results of the experiments are shown in table 3.

The data in table 3 indicate that the animals vaccinated with organisms irradiated for one-half or two and one-half minutes were partially protected against virulent inoculation. Not only was the mean survival significantly prolonged in both these groups, but one animal of the group vaccinated with organisms irradiated two and one-half minutes survived the virulent inoculation and showed no ill effects thereof

seventy-five days later. In the groups vaccinated with organisms irradiated five minutes and ten minutes, the mean survival was not significantly prolonged, but one animal survived in the group vaccinated with organisms irradiated five minutes. This result was considered

TABLE 3

Survival time in days following intracerebral inoculation of 0.000,01 mg. of virulent tubercle bacilli strain H37. Comparison of nonvaccinated controls with animals vaccinated with irradiated H37

VACCINATED WITH H37 IRRADIATION	SURVIVAL IN DAYS		VACCINATED WITH H37 IRRADIATION	SURVIVAL IN DAYS	
	Individual	Mean		Individual	Mean
<i>minutes</i>			<i>minutes</i>		
0.5	35	53.3 \pm 3.5	5	30	44.0 \pm 3.4
	37			37	
	41			39	
	53			51	
	58			59*	
	60				
	67				
	75†				
2.5	47	63.3 \pm 2.6	10	†	44.75 \pm 3.8
	55			32	
	56			43	
	58			49	
	69			59‡	
	72				
	75‡				
	75*				
Nonvaccinated controls	22	33.0 \pm 1.9	Nonvaccinated controls	28	40.4 \pm 2.6
	26			37	
	28			42	
	29			45	
	36			50	
	39				
	42				
	42				

* Animal in excellent condition. Sacrificed to end experiment.

† Animal succumbed to intercurrent infection.

‡ Animal paralyzed. Sacrificed.

significant because normal, nonvaccinated animals subjected to intracerebral inoculation of 0.000,01 mg. of this line of the strain H37 inevitably succumbed.

From experiments reported earlier in this paper (table 1) we know that the vaccines irradiated respectively one-half, two and one-half and five

minutes contained viable organisms, while the suspension irradiated ten minutes apparently contained none. Thus the three groups of animals which showed demonstrable protection were vaccinated with suspensions which contained viable organisms; and the group which exhibited no protection was vaccinated with a suspension containing no viable organisms. This result would seem to indicate that the radiation rendered the organisms nonviable and inactivated the immunizing capacity at about the same time. However, it is possible that one might irradiate a less dense suspension for a shorter period and render the organisms nonviable while maintaining the immunizing power.

Microscopical surveys of the lesions in the three groups of animals in the left half of table 3 revealed that the preparatory vaccinations influenced the histogenesis of lesions. Pulmonary tubercles were not found microscopically in any of the 8 controls, but there were lesions in the tracheobronchial lymph nodes of all save one. Each control exhibited extensive lesions in the spleen, liver and cervical lymph nodes, and massive, acute tuberculous meningo-encephalitis. The lesions of the spleen and brain showed many tubercle bacilli. In the vaccinated groups, on the other hand, the lesions in brain and meninges were considerably less extensive (sometimes minimal), more of the hard type of tubercle and contained fewer organisms. We have previously described and illustrated similar lesions in animals vaccinated by other methods (23). Lesions in cervical nodes of the vaccinated groups were also less extensive. But lesions in other viscera such as spleen and liver were as extensive as in the controls. Moreover, the vaccinated animals in a few instances exhibited pulmonary lesions, possibly due to the fact that they survived longer.

Thus the vaccinations with irradiated suspensions containing viable bacteria afforded partial protection, as expressed by significantly greater survival and by complete protection of a small number of animals; and vaccination also exerted an inhibitory influence on the evolution of the local cerebral and meningeal lesions, but exerted no favorable influence on the development of metastatic lesions. (It is possible that a part of the visceral lesions in the vaccinated animals may have been caused by the vaccinations.) An irradiated suspension which contained no viable organisms gave no evidence of protection against virulent inoculation.

DISCUSSION

The significant points brought out by this investigation are: first, that ultraviolet radiation may be so applied to tubercle bacilli that they are

rendered nonvirulent without being made nonviable; and second, that irradiated viable tubercle bacilli may induce demonstrable immunity in experimental animals.

Regarding the first point we believe that the reduction in virulence is probably an effect on the individual bacterial cell which precedes the lethal effect occurring with prolonged exposure to radiation. The only other apparent explanation of the effect would be to assume that the reduction in virulence is due to early death, during irradiation, of a large proportion of the bacterial population; but that the survivors are virulent. The results show that early death of a portion of the bacteria does occur; but the methods used to study virulence are so sensitive, and the dose of organisms used to test the virulence of irradiated suspensions was so large, that death would certainly have occurred had virulent organisms been present in the inocula. However, suspensions which failed to cause death when inoculated intracerebrally still contained organisms which grew in culture.

This latter point may have a bearing on the view held by some workers that bacteriological methods are superior to animal inoculations for detecting tubercle bacilli. It is possible that their opinions are based on obtaining in culture attenuated organisms which are incapable of inducing disease in animals.

Regarding immunization with viable irradiated organisms it must be emphasized that this procedure is not recommended for practical purposes as it would undoubtedly be associated with unwarranted hazards. In our experiments, organisms killed by irradiation did not induce demonstrable immunity. However, we used in these experiments dense suspensions which required long exposure to the lamp (ten minutes) to render the organisms nonviable. The heavy suspensions were used because we desired to have a given quantity of organisms in the irradiated suspension to be used as vaccine. This result could be accomplished by other methods, and the possibility remains that a weak suspension might be used in which very short exposure to radiation would cause death of the bacteria, conceivably without denaturing or rendering ineffective the immunizing antigen.

SUMMARY

The effect of approximately monochromatic ultraviolet radiation (2,537 Å) upon saline suspensions of human tubercle bacilli has been studied. The following effects on the viability, staining properties, virulence and immunizing power were observed.

Heavy suspensions of tubercle bacilli (1 mg. per cc.) require relatively long periods of irradiation (ten minutes or more) to be rendered nonviable. Weaker suspensions are killed in shorter time.

Organisms killed by ultraviolet radiation retain the property of acid-fastness.

Heavy suspensions of tubercle bacilli are rendered avirulent only after relatively long exposure to ultraviolet radiation; but weak suspensions are quickly reduced in virulence. Reduction in virulence can be demonstrated after less irradiation than is required to kill the organisms, and organisms may be made avirulent without being killed.

Irradiated viable organisms possessed the capacity of inducing demonstrable immunity. Organisms killed by the radiation did not induce measurable immunity.

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- (19) SMITHBURN, K. C.: *Ibid.*, 1936, 64, 771.
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BIOLOGICAL AND IMMUNOLOGICAL IDENTITY OF TOXOPLASMA OF ANIMAL AND HUMAN ORIGIN

By ALBERT B. SABIN

(From the Laboratories of The Rockefeller Institute for Medical Research)

There exists considerable confusion among parasitologists as to the characteristics which justify the classification of Protozoa of certain morphology as *Toxoplasma*. The capacity to multiply and to produce disease in a variety of hosts, including mammals and birds, must, in accord with the conclusions of Aragao,¹ be regarded as the chief taxonomic characteristics of the group. Morphology as the only guide can be misleading and confusing as is evident from the present controversy as to whether certain forms observed in avian malaria are *Toxoplasma* or stages of *Plasmodium*.² In accord with Aragao's criteria, the existence of *Toxoplasma* in North America was first demonstrated by Sabin and Olitsky,³ who isolated *Toxoplasma* from the brain of a guinea pig and showed them to be pathogenic for guinea pigs, rabbits, mice, monkeys and chickens. The forms seen by Mooser⁴ in guinea pigs in Mexico and by Markham⁵ in the United States may have been *Toxoplasma* but in the absence of transmission-experiments, the diagnosis remained uncertain. The reports by Manwell and Herman,⁶ Herman,⁷ and Wood and Wood⁸ of the presence of *Toxoplasma* in North American birds cannot be accepted as proved, since the identification was made only on morphologic grounds, while transmission to other birds was unsuccessful

¹ Aragao, H. de B., *Compt. rend. Soc. biol.*, 1933, **113**, 214.

² Hegner, R., and Wolfson, F., *Am. J. Hyg.*, 1938, **27**, 212; *ibid.*, 1938, **28**, 437.

³ Sabin, A. B., and Olitsky, P. K., *Science*, 1937, **85**, 336.

⁴ Mooser, H., *J. Infect. Dis.*, 1929, **44**, 186.

⁵ Markham, F. S., *Am. J. Hyg.*, 1937, **26**, 193.

⁶ Manwell, R. D., and Herman, C. M., *J. Parasitol.*, 1935, **21**, 415.

⁷ Herman, C. M., *Am. J. Hyg.*, 1937, **25**, 303; *Tr. Am. Micr. Soc.*, 1938, **57**, 132.

⁸ Wood, F. D., and Wood, S. F., *J. Parasitol.*, 1937, **23**, 197.

and no tests were made on mammals. The parasites identified by Wolfson⁹ as *Toxoplasma* in canaries were transmitted to other canaries but no tests with mammals were reported. Similarly it may be said that while human infection with *Toxoplasma* has been suggested by several investigators on morphological grounds, it has not hitherto been proved by adequate animal transmission and identification. The circumstances under which Bland¹⁰ obtained *Toxoplasma* in a rabbit after inoculation with blood from a patient suffering from glandular fever (infectious mononucleosis) were such that one could not be certain whether they originated in the human blood or the rabbit. In view of the demonstration that monkeys recovering from experimental toxoplasmosis developed neutralizing antibodies for the parasites,⁸ a number of sera from patients recovered from infectious mononucleosis were tested for such antibodies against our *Toxoplasma* and none was found.

In my opinion the first definite evidence that *Toxoplasma* can infect human beings has just been supplied by Wolf, Cowen, and Paige¹¹ with a case of encephalitis in a child. They not only demonstrated parasites of typical morphology in the human tissues but isolated *Toxoplasma* from a large number of rabbits and mice that were injected with the human brain. The fact that they used a large number of animals for transmission and that so many of them developed the infection almost simultaneously after a suitable incubation period leaves little doubt that the parasites originated in the human tissue. After several passages in mice these investigators submitted to this laboratory for a comparative study their *Toxoplasma* of human origin in the form of an infected mouse. As regards pathogenicity for a wide host-range, including mammals and birds, the *Toxoplasma* of human (Hum.) origin corresponded in every respect to those of animal (An.) origin. Mice injected intracerebrally (0.03 cc) and intraabdominally (0.5 cc) with infected mouse-brain died with nervous signs after incubation periods of 5 to 8 days. When the inoculation was made only intraabdominally, all mice became

⁹ Wolfson, F., *J. Parasitol.*, 1937, **23**, 553.

¹⁰ Bland, J. O. W., *Lancet*, 1930, **2**, 521; *Brit. J. Exp. Path.*, 1931, **12**, 311.

¹¹ Wolf, A., Cowen, D., and Paige, B., *Science*, 1939, **89**, 226.

sick, many with nervous signs, and the majority died while some survived with chronic disease and infection. The parasites seen in films of the peritoneal exudate, the brain, and viscera were morphologically identical with the ones studied in this laboratory for the past 4 years. Intracutaneous injection of 0.2 cc to 0.3 cc of infected mouse-brain suspension on the back of rabbits, was followed by the development (after 3 to 4 days) of a characteristic indurated skin-lesion, the center of which eventually underwent hemorrhagic necrosis, and of a cycle of fever of 5 to 8 days' duration, terminated by either death or recovery; of four rabbits inoculated in this manner, 2 died and 2 recovered. Not only were the cutaneous lesions and the clinical course indistinguishable from those induced by the "An." *Toxoplasma*, but pathologically there was also a striking similarity in the presence of necrotic foci in the viscera, especially the liver.

Two 1-day-old chicks (Rhode Island Reds) inoculated intracerebrally with 0.06 cc of a 10% suspension of infected mouse-brain developed nervous signs on the 5th and 6th days respectively. One died on the 6th day and the other was sacrificed when *in extremis*. *Toxoplasma* were demonstrated in stained impression-films of their brains and by passage to other chicks. Of two 3-weeks-old chicks inoculated intracerebrally with 0.1 cc of the same mouse-brain suspension, one exhibited transitory weakness and very slight incoördination on the 5th and 6th days, while the other showed no signs of disease; at the end of 4 weeks *Toxoplasma* were demonstrated in the brains of both these chicks by combined intracerebral and intra-abdominal inoculation of mice. Passage to other chicks was possible when the infected chick brain was injected intracerebrally but not intramuscularly (Table I).

The immunological identity of the *Toxoplasma* of animal and human origin was established by active cross-immunity and by neutralization-tests. Two rabbits that had recovered from an intracutaneous inoculation of "An." *Toxoplasma* were injected intracutaneously with "Hum." *Toxoplasma* along with 2 normal controls. The 2 convalescents remained well without developing either the typical skin-lesion or fever, while the controls contracted the characteristic disease. The same result was obtained when 2 "Hum." *Toxoplasma* convalescents were reinoculated with "An." *Toxoplasma*

along with 2 controls. Chart 1 shows the temperatures of one set of rabbits in each test. The neutralization-test was carried out with a hyperimmune monkey's serum prepared against the "An." *Toxo-*

TABLE I
Pathogenicity of Toxoplasma of Human Origin for Chicks

Experiment	Source of <i>Toxoplasma</i>	Route of inoculation	Dose, cc	Age of chicks	Chick No.	Result	Remarks
A	Mouse-brain suspension*	Intracerebral	.06	1 day	1	CNS 5th, dead 6th	<i>Toxoplasma</i> present in film of brain
					2	CNS 6th, sacrificed 6th	<i>Toxoplasma</i> present in film of brain. Brain-passaged to other chicks
			.10	3 wk	3	Slight CNS 5th, 6th; recovered	<i>Toxoplasma</i> in brain 4 weeks after inoculation demonstrated by mouse-passage
					4	No signs of illness	<i>Toxoplasma</i> in brain 4 weeks after inoculation demonstrated by mouse-passage
B	Chick-brain suspension	Intracerebral	.06	5 days	5	CNS 6th, dead 7th	<i>Toxoplasma</i> present in film of brain
					6	Slight CNS 7th, 8th; recovered	<i>Toxoplasma</i> in brain 4 weeks after inoculation demonstrated by mouse-passage
		Intramuscular	1.00	5 "	7	No signs of illness	Brains, lungs, and spleens injected in mice 1 month after inoculation; no <i>Toxoplasma</i> obtained
					8	" " " "	
					9	" " " "	

* Four mice inoculated intracerebrally with 0.03 cc of same suspension exhibited signs of encephalitis on 5th day and were dead on the 6th.

CNS 5th = Signs of encephalitis on 5th day.

plasma in 1935. A 10% suspension in Tyrode's solution of infected mouse-brain suspension was allowed to sediment spontaneously for a half hour; the supernate and dilutions prepared from it in Tyrode's solution were mixed with equal amounts of undiluted immune or

normal monkey's serum. After 10 minutes at room-temperature 0.2 cc of each mixture was injected intracutaneously on the back of a single rabbit. The results are shown in Chart 2.

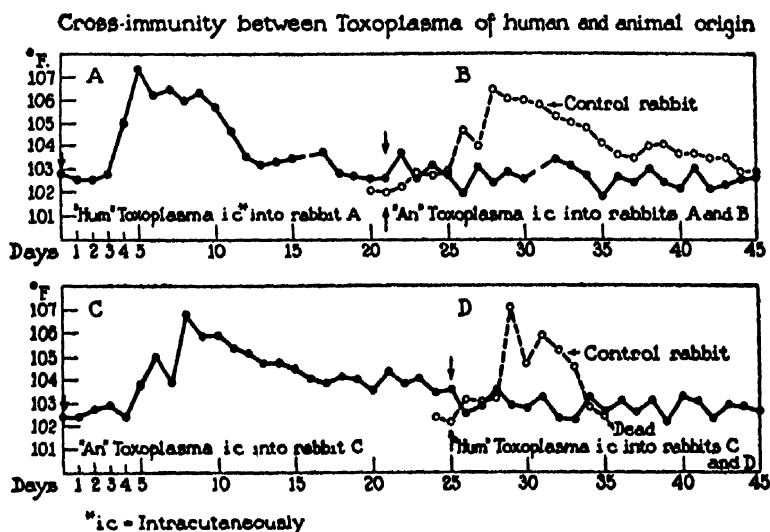


CHART 1

Neutralization of *Toxoplasma* of human and animal origin by serum of monkeys hyperimmunized with 'Animal' *Toxoplasma*

					Dilution of toxoplasma-infected tissue in mixtures			
					1:20	1:100	1:1000	1:10,000
'An'	Toxoplasma	+	normal monkey serum					N
'	'	+	'An' immune	'	N	N	N	N
'Hum'	'	+	'	'	N	N	N	N
'	'	+	normal monkey serum					

Mixtures injected intracutaneously on the back of a single rabbit. Resulting lesions traced on 12th day

= Necrosis N = No lesion

CHART 2—One-third actual size

The remarkable immunological and biological identity between the *Toxoplasma* of animal origin and the first strain of human origin

suggests that the same protozoön may operate in all susceptible mammals, a fact which must be considered in the epidemiology of toxoplasmosis. The incidence of toxoplasmosis in animals and human beings remains to be determined, and the existence of clinically inapparent or unrecognized non-fatal cases will very likely be found to play a definite rôle in the dissemination of the infection. These studies also suggest that unless the parasites of birds which resemble *Toxoplasma* morphologically, but are not pathogenic for or do not multiply in mammals, can be shown to possess some immunological relationship to the classical *Toxoplasma*, they should be included in a separate group.

Conclusions. *Toxoplasma* of animal and human origin have been shown to be identical biologically in their pathogenicity for mammals and birds, and immunologically by producing an active immunity against one another and by the fact that a serum against one neutralizes both.

MICE AS CARRIERS OF PATHOGENIC PLEUROPNEUMONIA-LIKE MICROORGANISMS

By ALBERT B. SABIN

(From the Laboratories of The Rockefeller Institute for Medical Research)

Two distinct strains of filtrable pathogenic microorganisms of the pleuropneumonia group have recently been isolated from the brains of mice and shown to possess tissue affinities of such a nature that they can produce in mice two experimental diseases which bear some resemblance to certain phases of rheumatic fever and rheumatoid arthritis in man.¹ Strain A induces a transitory, migratory polyarthritis, multiplies in the brain and in mesothelial cells of the pleura, pericardium and peritoneum, and produces a typical exotoxin which gives rise to choreiform signs. Strain B produces no such toxin, but has an almost specific affinity for the joints in which it gives rise to a chronic, progressive, proliferative, ankylosing arthritis. These two strains are biologically and immunologically distinct from each other, from *Pleuropneumonia bovis* and from pleuropneumonia-like microorganisms that have been isolated from rats in pure culture or in association with *Streptobacillus moniliformis*.² These findings clearly suggested the necessity of determining whether or not similar microorganisms could be isolated from patients with rheumatic fever and rheumatoid arthritis, and following this indication, Swift and Brown³ reported the isolation of pleuropneumonia-like microorganisms from acute rheumatic fever material.

The chief purpose of the present communication is to record certain experiences which indicate the inadvisability of using mice in attempting to isolate such microorganisms from human material. While

¹ A. B. Sabin, SCIENCE, 88: 575, 1938; *ibid.*, 89: 228, 1939.

² E. Klieneberger, *Jour. Hyg.*, 38: 458, 1938.

³ H. F. Swift and T. M. Brown, SCIENCE, 89: 271, 1939.

studying exudates and tissues from patients with rheumatoid arthritis or rheumatic fever, it was found that inoculation of such material, normal synovial fluid or sterile broth into the eyes (vitreous) of mice, yielded positive pleuropneumonia-like cultures with great regularity. In a typical test, material under investigation was injected into both eyes of six 3-weeks-old mice; six days later the eyes were removed with separate, sterile instruments, immersed in anesthetic ether for one to two minutes (this was sufficient to bring about adequate sterilization of the exterior of the eye), incised, and streaked across 30 per cent. ascitic fluid agar. In most instances, innumerable, typical, microscopic, pleuropneumonia-like colonies appeared within two days. With the Rockefeller Institute Swiss stock, at least four or five mice out of each group of six yielded positive cultures from one or both eyes in ten different experiments. The colonies on solid medium resembled those of Strain A; after several transfers on fluid medium the cultures were found to be agglutinated in high titre by anti-A serum and to produce a neurotoxin which was completely neutralized by the "A" strain antitoxin. Further studies revealed that these microorganisms were inhabitants of the external surface of the eye, from which they could be cultured with great regularity, and frequently in large numbers unassociated with any bacterial colonies. It was apparent that as a result of the intraocular injection some of these microorganisms were carried into the eye, where they multiplied in the course of a few days. Similar studies carried out with mice of the Rockefeller Institute albino stock and of another Swiss strain (Freed) originating from the Institute stock but bred elsewhere for the past six to seven years, revealed the same condition, but the carrier rate was lower; it was thus possible to encounter groups of six mice from which these microorganisms could not be obtained, while in other groups of six, either all or a varying number yielded positive cultures of the same type. Evidence was also obtained that these microorganisms inhabit the mucosa of the nose and accessory sinuses from which they may be carried into the lungs in the course of nasal instillation under anesthesia.⁴ It may be of interest to note here

⁴ This finding suggests that viruses, such as that of influenza, which are passed by nasal instillation in mice, should be cultured periodically to determine whether or not they have been contaminated by microorganisms of the pleuropneumonia group.

that in one test in which six mice were given nasal instillations under ether anesthesia and intraocular injections of pericardial fluid from a patient who succumbed to acute rheumatic carditis, three different types of pleuropneumonia-like microorganisms were isolated: a Type A from the eyes, a Type B from the lungs of one mouse, and a new type (to be called "C") which produced arthritis in mice but no toxin and was immunologically distinct from all the others studied. Two pleuropneumonia-like strains producing pneumonia in mice (isolated by Dr. Dienes in the course of passing two human rheumatic heart muscle suspensions through the lungs of mice) were submitted to me for study⁵; they were found to be immunologically identical with Strain A and to produce neurotoxin in cultures which was neutralized by "A" antitoxin. Of three cultures isolated by Drs. Swift and Brown from pneumonic lungs of mice inoculated with rheumatic fever material, one was found to be a Type A, one a Type B and the third the same as the newly isolated Type C.

It is apparent, therefore, that the presence or absence of pleuropneumonia-like microorganisms in rheumatic fever and rheumatoid arthritis exudates and tissues will have to be established primarily by cultural methods, and not by passage through mice or other animals. Using a large variety of solid and fluid media and "passaging blindly" six or more times, I have been unable thus far to grow pleuropneumonia-like microorganisms from thirteen rheumatoid arthritic exudates (twelve patients), four rheumatoid subcutaneous nodules (three patients), rheumatoid synovial tissue (two patients), acute rheumatic blood, pleural fluid, pericardial fluid and heart muscle (two patients),⁶ but many more cultivation experiments will have to be performed with suitable specimens before a final decision can be reached.

⁵ Dr. L. Dienes informs me that he was able to isolate a similar strain by passaging normal mouse lung.

⁶ I am deeply indebted to Dr. Edward F. Hartung, of the New York Post-Graduate Hospital, for supplying most of these specimens.

PROTECTIVE ANTIBODIES AGAINST EQUINE ENCEPHALOMYELITIS VIRUS IN THE SERUM OF LABORATORY WORKERS

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(From the Laboratories of The Rockefeller Institute for Medical Research)

It has been shown recently¹ that a large proportion of mice and guinea pigs develop, with increasing age, physiological or structural barriers that prevent certain viruses from invading the central nervous system. This resistance is demonstrable when virus is given peripherally, as, for example, intraabdominally or intramuscularly, but not when it is injected directly into the brain. It is not a result of prior infection nor is it associated with the presence of protective substance in the serum. Furthermore, in the recent epidemic of equine encephalomyelitis (E. E.) in man in southeastern Massachusetts,² children were predominantly affected. The older animals which resist the E. E. viruses develop systemic infection, as is evidenced by the finding of virus in the circulation and later the presence of protective antibodies in the serum. In view of this suggestive relationship of age of both the experimental animal and man to clinically apparent infection with this virus, it was thought desirable to undertake a study of the protective capacity of the serum in certain individuals in our laboratory, who had been in contact with the E. E. virus for a period extending from 1 to over 6 years. The results would indicate whether a clinically inapparent infection, as determined by the presence of protective antibody, could possibly have occurred during that time.





















Serum-protection tests were carried out in mice by the intra-

¹ Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1937, **66**, 15, 35; 1938, **67**, 201, 229; Sabin, A. B., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 270; Sabin, A. B., and Olitsky, P. K., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **38**, 595, 597.

² Fothergill, L. D., Dingle, J. H., Farber, S., and Connerley, M. L., *New England J. Med.*, 1938, **219**, 411; Webster, L. T., and Wright, F. H., *Science*, 1938, **88**, 305; Feemster, R. F., *Am. J. Pub. Health*, 1938, **28**, 1403; Wesselhoeft, E., Smith, E. C., and Branch, C. F., *J. Am. Med. Assn.*, 1938, **111**, 1735.

abdominal method of Olitsky and Harford;³ that is, by injecting, by the intraabdominal route, 0.03 cc of a mixture of equal parts of test serum and virus-dilutions (tenfold dilutions were used) into 15-day-old mice. Each mixture was given to groups of 3 or 4 mice. The Eastern strain of virus (E. E. E.), was derived from a stock which

Mouse protection test
with patient's serum

Final dilution of virus	EEE		WEE	
	Test serum	"Normal" serum H	Test serum	"Normal" serum D
10^{-2}				
10^{-3}				
10^{-4}				
10^{-5}				
10^{-6}				
10^{-7}				
10^{-8}				

 1 mouse survived

 1 " died

FIG. 1



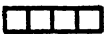






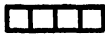
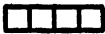


was frequently passaged in mouse brain and was again passaged through mouse brains immediately before use.

Two human sera, H and D, considered as "normal," were employed. The first was obtained from a worker in this laboratory in 1931 before the virus had been introduced here for study; the second was collected in January, 1938, and had been previously shown to afford no protection against E. E. E. virus. The titer of the virus in

³ Olitsky, P. K., and Harford, C. G., *J. Exp. Med.*, 1938, **68**, 173.

the control series, with or without these sera, was 10^{-7} or 10^{-8} (Figs. 1, 2, and 3). Another serum was added* which was obtained from a patient who 5 weeks previously had become acutely ill and had developed encephalomyelitis followed by recovery. This patient had been engaged in the procedure of inoculating E. E. virus in chick embryos. The serum, after the acute illness, protected mice against

**EEE mouse protection test
with human sera**

Final dilution of virus	Test sera			"Normal" serum
	A	B	C	D
10^{-3}				
10^{-4}				
10^{-5}				
10^{-6}				
10^{-7}				
10^{-8}				
10^{-9}				

Symbols as in chart 1

FIG. 2

100,000 minimal infective intraabdominal doses of E. E. E. virus by the intraabdominal test and showed no protection against the Western strain (W. E. E.) (Fig. 1).

Of the 6 sera collected from the individual members of our laboratory, 5 revealed no protective antibodies against E. E. E. virus (Figs. 2 and 3); variations are not significant, as shown by the controls. On the other hand, one (A) protected against 1,000 to 10,000 intra-

* We express with pleasure our deep obligation to Dr. J. H. Warvel of Indianapolis, Indiana, for this material and the history of the case.

abdominal lethal doses. The latter was obtained from a person who has been associated with work on E. E. virus (mostly the Eastern strain) for 6 years; at no time has he passed through an illness resembling encephalomyelitis and his general health has remained excellent. None of the 6 sera showed protective antibodies against the W. E. E. strain.

In view of the recently presented hypothesis that localized barriers develop with increasing age, or are present in particular hosts,¹ which prevent certain viruses from invading the CNS, the positive result

**EEE mouse protection test
with human sera**

Final dilution of virus	Test sera			"Normal" serum	Broth control
	E	F	G	D	
10^{-6}	██████				██████
10^{-7}	██████	██████	██████	██████	██████
10^{-8}	██████	██████	██████	██████	██████
10^{-9}				██████	██████

Symbols as in chart 1

FIG. 3

herein reported of the presence of protective antibodies in an adult person who has been exposed to the virus in the laboratory, takes on added interest. A suggestion offered⁴ is that in man, if the pattern of viral invasion from the periphery to the CNS follows that in the mouse or guinea pig, then the probability exists that in most human adults the virus may perhaps be prevented from invading the CNS by certain localized barriers. Hence adult contacts during an epidemic may have clinically inapparent infection and possibly reveal virus in the circulation. In such instances protective antibodies may be found later in the serum. Proof of this assumption would, of course, depend on further observations in the field.

⁴ Sabin, A. B., personal communication.

CELLULAR REACTIONS TO A DYE-PROTEIN WITH A CONCEPT OF THE MECHANISM OF ANTIBODY FORMATION

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PLATE 7

(Received for publication, April 6, 1939)

It has long been known that antibodies are almost invariably associated with the globulin fractions in the serum. An example of this association in the case of anti-pneumococcus serum was presented some years ago by Avery (1). Furthermore, within the last decade, evidence that antibodies are themselves protein has been steadily accumulating. Especially convincing have been the studies of Felton and his collaborators (2, 3) on the concentration and purification of the antibodies in antipneumococcus horse sera and the extension of these studies by Chow and Goebel (4). However, without quantitative micro methods for the estimation of antibodies in absolute terms the identification of antibodies as modified serum globulins would necessarily have remained uncertain. The development of such methods by Heidelberger and his collaborators (5) and the extension of their theoretical studies to the preparation of more highly purified antibody than had previously been obtainable led to accurate investigations on the physical-chemical properties of antibodies by the ultracentrifugal (6-9) and electrophoretic (10, 11) methods. Since the molecular weights and electrical mobilities found were those characteristic of proteins, the identification of antibodies as modified serum globulins may be considered accomplished. A chemically satisfactory theory of antibody formation has been put forward by Breinl and Haurowitz (12) and was restated by Mudd (13).

All of this chemical work signifies that the cellular mechanisms which give rise to antibodies must be concerned in their normal functions with the synthesis of globulin. For some years evidence has been presented implicating the cells of the reticulo-endothelial system in the formation of antibodies. The present report seeks to make our understanding of the mechanism of this function more definite.

Studies of the reactions of the phagocytic mononuclear cells stem from the work of Ehrlich and Metchnikoff. It is known that when Ehrlich was experimenting with dyes, he suggested to his friend Goldmann that he use pyrrol blue for observations on these cells. Goldmann (14) then made a survey of all the cells in the body which will phagocytize azo dyes given in the form of particulate matter. His work was followed by that of Aschoff (15) and of Kiyono (16) who used carmine particles, and by that of Evans and his associates (17-19) who employed trypan blue. Aschoff formulated the concept

of the reticulo-endothelial system. Though this name has been used in different ways, the idea is based on the fact that certain specific endothelia and certain mononuclear cells of the tissues have high phagocytic power and react toward the same materials. With these studies as a basis there has grown up an extensive literature implicating the reticulo-endothelial system in the formation of antibodies. It includes observations involving the spleen (20), the lymph nodes (21), and macrophages or clasmotocytes (22-24). A critical review of this literature has been given by Zinsser, Enders, and Fothergill (25).

In studies with chemical fractions from tubercle bacilli it was found that certain lipids, notably a phosphatide (26) and wax-like substances (27), could be identified within the phagocytic cells, the phosphatide because it assumed the form of myelin figures, and the wax because of its acid fastness. This made it possible both to identify with assurance the cells which had reacted to these materials and to follow to some extent the ability of these cells to deal with them. The wax was seen to become surrounded by monocytes which fused into giant cells to engulf it; within these giant cells the wax soon lost its acid fastness and was then slowly degraded until the cytoplasm of these cells returned to the normal state, the giant cells splitting into the original monocytes (27). On the other hand, single monocytes engulfed the phosphatide without any fusion of cells; occasional multinuclear forms were induced by the multiplication of nuclei without cell division. The degradation of the phosphatide appeared to be only partial for an epithelioid cell was formed which never returned to the size and appearance of the monocyte. These epithelioid cells then died either singly or *en masse*, bringing about a form of caseation (26). These examples illustrate two methods of dealing with phagocytized material, the one instance showing what may be called complete digestion of the material, the other, a partial digestion with permanent storage of some intermediate product. Still another mechanism involving the phagocytosis of an antigen will be considered.

Many recent studies on the origin of the serum proteins have also been pointing, in our judgment, toward the reticulo-endothelial system. Most of these studies have implicated the liver, but have also suggested that the liver is not the sole source of these proteins. The involvement of the liver is due, it would seem, to the presence of the Kupffer cells, a part of the reticulo-endothelial system. Observations on the liver have been made largely after damage of the organ by chloroform, or the use of an Eck fistula, or after total extirpation of the liver (28-37). Other observers have specifically implicated other tissues as well as the liver (38, 39).

In 1912, Downey and Weidenreich described and illustrated as a characteristic of lymphocytes a shedding of parts of the surface films or exoplasm of these cells, which caused no permanent damage to the cells. They demonstrated this phenomenon in lymphocytes within the sinuses of lymph nodes and also in lymphocytes throughout the follicles (40). Later Downey (41) studied the phenomenon further and showed that the shed bits of cytoplasm were not platelets, since they were devoid of the granules which characterize platelets. He observed that they disintegrated rapidly in the thoracic duct.

This phenomenon has been observed in our laboratory as a characteristic of monocytes (macrophages or clasmotocytes) after they have been stimulated and it has been illustrated after injections of tuberculo-protein (Fig. 8 in reference 42). As a matter of fact, it occurs in the normal reaction of monocytes or macrophages and was observed many years ago by Ranvier (43). He described such shedding of exoplasm from certain

connective tissue cells in the frog and on account of this property named the cell "clasmatocyte." Since that time the terms macrophage ("big eater") and clasmatocyte (shedding of exoplasm) have been used synonymously and thus have been recorded two of the properties of the same cell.

The synthesis of a dark red dye-protein, R-salt-azo-benzidine-azo-egg albumin, by Heidelberger and Kendall in 1930 (44-46) provided a material which can also be positively identified within cells, in this instance by its color. Moreover, this material has the added important property of being a good antigen. They employed the substance in a study of the quantitative relations in the antigen-antibody precipitate, since the amount of antigen could be determined colorimetrically both in the precipitate and in the supernatant. In synthesizing this antigen Heidelberger had also in mind that it might be an effective material for following the cellular reactions involved in sensitization and in immunity.¹ It has been proven that the dye-protein is also taken up by the cells of the reticulo-endothelial system and the following pages will describe what may be observed concerning the reactions of the phagocytic cells to this antigen.

In 1920 the author published studies on the origin of blood vessels in the classic material of the chick blastoderm, using the method of mounting the living blastoderm in a hanging drop preparation and watching the development of blood vessels for some hours. In such preparations of chicks of the 2nd day of incubation it was possible to see the origin of a vessel by the liquefaction of the central part of a mass of angioblasts while the peripheral cells flattened out to make endothelium (see Fig. 23 in reference 47). This process had been inferred years before from a study of sections by Klein (48). The phenomenon may be stated in physiological terms as follows: Since for a vessel to function as such, it is essential for the plasma to contain some protein to maintain the balance of intra- and extravascular fluid, the original transformation of a solid mass of angioblasts into a vessel involves the sacrifice of some cytoplasm to make the serum proteins. In the present studies some evidence will be presented in support of the concept that throughout life the serum proteins, certainly as far as globulin is concerned, come from the sacrifice of a part of the cytoplasm of cells. This process has no relation to the function of secretion, in which case a material, formed by the cytoplasm but clearly distinct from it, appears within the cell and is cast out of it with no loss of cytoplasm whatever. If it be correct that the Kupffer cells of the liver sinuses play a part in this process, it is interesting to record that Kupffer cells develop in the embryo long before blood islands have entirely disappeared. This ob-

¹ We are indebted to Dr. Heidelberger not only for giving us the material for our experiments but also for making all of the quantitative estimations of the antibody titre in the sera and for help and advice throughout the work.

servation was checked in sections of a monkey, perfectly preserved, at the Carnegie Institution of Embryology, through the courtesy of Dr. George L. Streeter (monkey embryo 479, 12 mm., ovulation age, 35 days).

RESULTS

The dye-protein was used in the form of an alum precipitate, since Heidelberger, Kendall, and Soo Hoo (44-46) had found that with this form the antibody titre was higher than after the same material given in solution. It was a suspension of purplish red particles which settled quickly on standing and were in aggregates large enough to be readily visible. The material was introduced by four routes, namely, the intraperitoneal, intravenous, intradermal, and subcutaneous. After intravenous injections the dye-protein-alum particles were found in the Kupffer cells of the liver, in the macrophages of the splenic pulp, and, to a minor extent, in the macrophages (the so called adventitial cells) which lie along the border of the sinusoids in the bone marrow. After intraperitoneal injection the dye-protein was found in the macrophages of the milk spots of the omentum and in the corresponding cells of the peritoneal walls, as well as in the endothelium lining the lymphatic sinuses of the retrosternal nodes and in the free macrophages of sinuses and follicles of these nodes. After intradermal and subcutaneous injections the dye-protein was in the local macrophages and in the regional lymph nodes. After a single subcutaneous injection macrophages engorged with dye were not found in the subcutaneous tissues but only in the regional lymph nodes.

The dosage, route, and spacing of the injections, together with the cellular reactions and the antibody titre of the serum, are presented in Table I. Many additional subcutaneous injections into the tissues of foot pads and lips were made but the results are not recorded in this table.

The intraperitoneal route of injection stimulates tissues that lend themselves most readily to the study of the living cell in films of the omentum, in peritoneal exudates, and in the tissues of the retrosternal nodes. In Fig. 1 is shown a part of a milk spot of the omentum of rabbit R 5109, 24 hours after a final injection of 2 mg. of the dye-protein. As will be noted in Table I, this animal had received 50 mg. of the material intraperitoneally, the last injection having been made 24 hours before the tissues were studied. The macrophages of the milk spots were heavily loaded with particles of the dye-protein, as demonstrated by their color in the photograph. These cells were studied while alive and with no stain whatever, so that the dye-protein could be positively identified. The film was then fixed and the nuclei were stained in hematoxylin only, care being taken that

all other fluids used did not contain any counterstain. All of the red granules in the photograph are due, therefore, to the material injected. In the macrophages in Fig. 1 the numerous particles of dye-protein are plainly visible, but there are also one or two larger vacuoles which in the living cell were translucent and smooth in contour, as if of fluid, in contrast to the smaller, irregular, opaque particles of the dye-protein-alum-salt. These larger vacuoles are interpreted as containing dye in solution which the cell had been able to separate from the dye-protein and had segregated into a few vacuoles by itself. The particles of the residual dye-protein varied in the density of color according to the varying amounts of dye which had been extracted from each. This variation in the density of the color is imperfectly recorded in the photograph. In the lower macrophage of the figure are three or four blue dots which are the residue of the nucleus, probably nucleic acid of a neutrophile which had been phagocytized. These cells illustrate the first reaction of the phagocyte toward the engulfed material. Such materials are segregated into vacuoles of digestion, with a barrier of fluid between the ingested material and the cytoplasm. This barrier of fluid can be made visible by treating the living cell with neutral red, which procedure stains the digestive fluid and then conceals the phagocytized particle. The first reaction of the cell was to separate some of the dye from each dye-protein aggregate. Besides being in the macrophages of the milk spots of the omentum, there was some dye-protein in a part of the fibroblasts between the milk spots. No fibroblast was as heavily loaded with the dye-protein as were the macrophages, but they also showed an occasional vacuole containing what has been interpreted as dye alone.

The intravenous injections provided material that was especially instructive. Rabbits H-668, 669, and 670 received 88 mg. of the dye-protein intravenously and were then tested after an interval of 1 week for antibodies in the serum. H-669 showed a relatively high titre, namely, 0.63 mg. N per cc., while H-668 had an intermediate titre, 0.30 mg., and H-670 a lower one, 0.27 mg. To this rabbit (H-670) was given a final intravenous injection of 8 mg. of dye-protein and the tissues were studied 6 hours later. The dye-protein was in all instances in the Kupffer cells of the hepatic sinuses, in the macrophages of the splenic pulp, and, to a minor extent, in the adventitial cells along the sinuses of the bone marrow. In rabbit H-670 many of the Kupffer cells along the portal border of the lobules of the liver were enlarged and engorged with the dye-protein, as revealed in Figs. 2 to 4. In Fig. 2 is shown a long Kupffer cell which contained, besides much dye-protein, a neutrophile, a red cell, and some nuclear debris. In one place the cytoplasm of this Kupffer cell stretches completely across the

TABLE I

Correlation between Cellular Reactions and Antibody Titre after Injections of an Alum-Precipitated Dye-Protein into Rabbits

Animal No.	Number, spacing, amount, and route of injections	Total amount of dye-protein mg.	Cellular reactions *	Antibody titre of serum
R 5108* R 5413 R 5414† R 5415†	12 and 14 daily 0.1 cc. of 5 per cent solution intradermally		The dye-protein remained visible through the skin and there was no sensitization	—
R 2714	4 daily 1 mg. intraperitoneally. Studied 4 days later	4	Omentum: Dye-protein in a few macrophages; some in neutrophils. Neutrophils containing dye within macrophages Retrosternal nodes: Dye-protein within lymphatic endothelium; monocytes and macrophages loaded with dye-protein	None
R 2817 R 2818	3 daily 1 mg. intraperitoneally. Studied 24 hrs. later	3	Omentum: Small amount of dye-protein in macrophages. Many neutrophils Retrosternal nodes: Endothelium and macrophages filled with dye-protein. Some neutrophils containing dye-protein. Dye-protein in small, irregular, opaque masses. Some macrophages had one round, red globule darker than the dye-protein	R 2817 none R 2818 not tested
R 2808 R 2433	3 every 4 days 1 mg. intraperitoneally. Studied 4 days later	3	Omentum: Some macrophages contained small amount of dye-protein Retrosternal nodes: Same The monocytes and macrophages of omentum and peritoneal exudates which no longer contained visible dye-protein showed shedding of surface films (Fig. 8)	R 2808 0.1 mg. precipitin per cc. R 2433 0.16 mg. precipitin per cc.
R 2765	4 daily 0.1 mg. intraperitoneally 5 daily 0.1 mg. intraperitoneally. Interval of 11 wks. 3 daily 1 mg. intraperitoneally. Studied 4 days later	3.9	Omentum: Traces of dye-protein in macrophages Retrosternal nodes: A few macrophages containing dye-protein and dye only	After first 2 courses too little precipitin for accurate estimation. 4 days after final injection 0.47 mg. precipitin per cc.
R 2503	4 daily 10 mg. intraperitoneally. Studied 2 days later	40	Omentum: Macrophages loaded with dye-protein in large aggregates. Many cells contained dye within neutrophils Retrosternal nodes: Same	—

* These are serial numbers used in this laboratory over a term of years.

† Guinea pigs.

TABLE I—*Concluded*

Animal No.	Number, spacing, amount, and route of injections	Total amount of dye-protein mg.	Cellular reactions	Antibody titre of serum
R 2636	2 mg. foot pads. Studied 4 hrs. later	2	Foot pads: Dye stained the fibers of the tissues. No dye-protein found in macrophages Popliteal nodes: Macrophages containing dye-protein	—
R 5109	6 weekly series 4 days each 2 mg. intraperitoneally. Interval of 12 days 2 mg. intraperitoneally. Studied 24 hrs. later	30	Omentum: Macrophages loaded with dye-protein. Some showed 2-3 large, round vacuoles darker in color (Fig. 1). Some dye-protein in fibroblasts Retrosternal nodes: Dye-protein in endothelium of sinuses. Macrophages loaded with it	Antibody titre weak after 3rd and 4th series. 11 days after 6th series, 0.07 mg. antibody N per cc. 24 hrs. after final injection, 0.04 mg. antibody N per cc.
R 5110	3 weekly series 4 days each 2 mg. intraperitoneally 3 weekly series 4 days each 2 mg. intravenously. Interval of 12 days 2 mg. intraperitoneally. Studied 24 hrs. later	50	Omentum: Macrophages contained some dye-protein Retrosternal nodes: Macrophages contained considerable dye-protein Liver: A few Kupffer cells contained little dye-protein Spleen: Many large macrophages with dye-protein and neutrophils	Antibody titre weak after 3rd series. 6 days after 6th series, 0.13 mg. antibody N per cc. 24 hrs. after last injection, 0.07 mg. antibody N per cc.
H-668 H-669	2 mg. intravenously Interval of 2 days 2 mg. 1 day 3 mg. 3 days. Interval of 3 days 4 mg. 1 day 5 mg. 3 days. Interval of 2 days 5 mg. 4 days. Interval of 2 days 6 mg. 2 days 8 mg. 2 days. Interval of 2 days 8 mg. 1 day. Studied 7 days later	88	Liver: Some dye-protein in Kupffer cells throughout lobule. All Kupffer cells normal size. Less dye-protein in cells of H-669, in which it could be found only with oil immersion lenses (Fig. 6) Spleen: Some dye-protein and debris of neutrophils in macrophages—less in H-669 Bone marrow: Traces of dye-protein in adventitial cells	H-668: 0.30 mg. antibody N per cc. H-669: 0.63 mg. antibody N per cc.
H-670	Same as above, except a final 8 mg. intravenously. Studied 6 hrs. later	96	Liver: Kupffer cells along portal border enlarged and engorged with dye-protein. They contained also some neutrophils, themselves containing dye-protein. Some monocytes containing dye in vessels Spleen: Macrophages engorged with dye-protein and neutrophils. Monocytes and neutrophils containing dye Bone marrow: Traces of dye-protein in adventitial cells Blood vessels: Monocytes and neutrophils containing dye-protein	0.27 mg. antibody N per cc.

lumen of the sinus. In addition to this endothelial cell is a monocyte engorged with dye-protein within the vessel. In Fig. 3 are shown two Kupffer cells chosen to demonstrate the folds of the surface films of these cells that serve as guy-ropes which fasten themselves to the opposite wall and keep these surface films floating in midstream to perform their function of phagocytosis. The surface films of the Kupffer cells, which are the most highly developed of any of the cells of the reticulo-endothelial system, have been shown in the isolated cell by Rous and Beard (49, 50). In Fig. 4 is revealed a multinucleated Kupffer cell containing dye-protein with a long film of cytoplasm which appears to be separated from the main mass by the plane of the section. These very large Kupffer cells were all near the portal border, while smaller ones were found more deeply placed, as in Fig. 5. This cell was chosen to show two engulfed neutrophils which themselves contain dye. In this animal many of the neutrophils in the vessels, for example, in the vessels of the kidney, contained dye. This phenomenon was especially prominent in the tissues of the spleen. The sections of the spleen demonstrate many neutrophils, monocytes, and macrophages filled with the dye-protein within the pulp. The macrophages for the most part contained both dye-protein and neutrophils. This reaction may be seen in Fig. 7.

The tissues of rabbits H-668 and 669 contained dye-protein in the cells and showed the same distribution of it except that it was far less in amount since they had not received a recent injection. These two animals had some residual dye-protein in both liver and spleen in inverse proportion to the antibody titre of their sera. The difference in the amount of dye-protein is sufficiently marked so that the sections may be discriminated without reference to their labels, for in the one, H-669, the dye could be located only with the oil immersion lens, while in rabbit H-668 it could still be discerned with low power, that is, with a 16 mm. lens. Traces of the dye-protein were visible in cells throughout the liver lobule, even in the Kupffer cells that are nearest to the central vein. The amount of dye remaining can be seen in Fig. 6, taken from the liver of rabbit H-669, in the cells at the extreme right and left of the photograph.

This marked reduction in the amount of dye-protein in some of the cells and its disappearance from most of the phagocytic cells were correlated with the appearance of antibodies in the serum. This correlation was present also in rabbit R 2433. This animal had received three spaced injections of 1 mg. of the dye-protein and the tissues were studied 4 days after the third injection. The mononuclear cells of the peritoneal exudate and the macrophages of the omentum showed a marked shedding of parts of

the surface films or exoplasm. Some of the macrophages had some residual dye-protein but it was striking that it was not these cells that presented the shedding phenomenon but rather those without visible dye-protein particles. This observation was quite clear in the preparations of the living cells studied without any accessory stain; the use of neutral red accentuated somewhat the phenomenon of shedding. Two cells from the peritoneal exudate of this animal stained in neutral red are shown in Fig. 8. The magnification at which the photograph was taken ($\times 1,800$) proved to be too high to demonstrate the processes of exoplasm to the greatest advantage; they are better illustrated at a magnification of 1,000 in the omentum of a rabbit obtained 7 days following an injection of tuberculo-protein (Fig. 8 in reference 42). It should be made clear that this process of the shedding of parts of the exoplasm has not been observed within the living animal, and thus not from the Kupffer cells, but only in living cells removed from the animal. When the omentum or a drop of peritoneal fluid is first mounted on a slide, all the cells are rounded, but on standing for a short time the reaction of the shedding begins by the pushing out of such processes of cytoplasm as are photographed in Fig. 8. These then separate from the cell and break into globules. It should be noted that the peritoneal exudates are mounted in their own fluid and the preparations are sealed immediately, so that there is little chance for any change in the surrounding fluid medium to produce this reaction. The omentum can only be mounted with the addition of saline. The one tissue in which the shed globules can be seen immediately upon removal from the body is that of scrapings from the regional lymph nodes, in which case, at the proper functional stage, the fluid of the sinuses is filled with these particles.

DISCUSSION

The data presented in this paper may thus be summed up as follows. The use of a "marked antigen," such as an alum-precipitated dye-protein, makes it possible to identify the cells by which it is phagocytized. The material is placed in the vacuoles of digestion by the cell and altered first by the removal of the dye. After removal of the dye the solid particles of protein disappear and it is assumed that the protein has been rendered into soluble form (possibly, as Heidelberger suggests, with amino groups replacing the original RN:N linkages) and passed into the cytoplasm. It should be stated here that Heidelberger has found that in the case of this antigen the antibodies are not oriented to the hapten group. Coincident with the time when the dye-protein is no longer visible within these cells, and when there are antibodies in the serum, there is a marked shedding of

the surface films of the macrophages without damage to them. The phenomenon of shedding of surface films is characteristic of the normal monocyte or macrophage; it occurs also after the phagocytosis of non-antigens, but it is much accentuated at certain periods after the ingestion of antigens. Thus these mononuclear cells function first as macrophages and then as clasmatocytes. The hypothesis which may be formulated from these observations is that the cells of the reticulo-endothelial system take up foreign materials which may be classified into two groups, namely, antigens and non-antigens. Both kinds of material are first taken into the vacuoles of the cells, indicating that a cell guards its basic cytoplasm from the immediate entrance of foreign substances. The vacuoles are the cellular organs of digestion; the cytoplasm is the zone of syntheses. In turn, the synthesis of cytoplasm is usually from normal food substances. If the material phagocytized is an antigen, it is rendered into suitable soluble form within the vacuole and then passed into the cytoplasm itself. There its presence in some way increases the synthesis of globulin and modifies some of it into antibody globulin (*cf.* 11, 12). With the shedding of parts of the surface films of these cells, both normal globulin and antibody globulin are carried into the blood plasma, since immunologists have discovered that both usually increase at the same time. Thus an antigen may be defined as a substance which can specifically modify the synthesis of cytoplasm. This process is the evolution of a change in cytoplasm in response to environment. It may be possible that the cell which has formed a new kind of globulin and still retains it in the cytoplasm is sensitized, meaning that it would react differently from the normal cell in the presence of the original antigen. If this be true, then certain phases of sensitization and immunization are founded on the same mechanism.

Certain details concerning the action of the phagocytic mononuclear cells illustrated in these experiments seem worthy of mention. A question has been raised concerning the name reticulo-endothelial system (24) *versus* the use of some term such as the macrophage system or the functions of the phagocytic mononuclear cells. The data obtained in this study illustrate the fact that it is not possible to introduce particulate matter by any of the four usual routes without involving both certain specific endothelial cells and free macrophages. After intravenous injection the endothelial cells are the Kupffer cells of the liver and the macrophages are those of the spleen and bone marrow. Following any kind of interstitial injection, intraperitoneal, subcutaneous, or intradermal, the material introduced is phagocytized by both the local macrophages and the lymphatic endothelium of the regional lymph nodes, together with macrophages in these nodes.

There is a marked contrast between the dermis and the subcutaneous levels in the abundance of macrophages in the one and their paucity in the other; macrophages may, however, be induced in the subcutaneous level. It may thus be seen that the reticulo-endothelial system is widespread and that phagocytic endothelium, either vascular or lymphatic, is always called into play together with free macrophages. Macrophages or their less mature form, monocytes, arise throughout the connective tissues of the body. From these observations it may be concluded that the reticulo-endothelial system has an enormous factor of safety in the sense discussed by Meltzer (51). This is clear also from the fact that after repeated intravenous injections not all of the Kupffer cells had engulfed the dye-protein and the same was true of the cells of the milk spots of the omentum. Moreover, it is well known from experiments with benzidine dyes (15, 16) that after repeated intravenous injections the endothelium of splenic and marrow sinuses becomes phagocytic. A further reservoir of phagocytic cells is brought about by the ready multiplication of monocytes in the tissues. This phenomenon was striking in experiments with tuberculo-protein (42) but there was no evidence of multiplication of cells following administration of dye-protein.

It is thus clear that for the experimental production of antibodies one may call into action either the tissues of the liver and spleen by employing the intravenous route of injection, or local macrophages and the endothelium and macrophages of the regional lymph nodes. As has been said, the production of antibodies within lymph nodes was proved by McMaster and Kidd in 1937 (21). In the present experiments a higher titre of antibodies was produced by the intravenous route with the use of the dye-protein. This is illustrated in the comparison of the antibody titres of rabbits R 5109 and 5110 (Table I). The total amount of antigen given was the same for these two animals, but R 5110 received 24 of the 50 mg. intravenously and had the higher titre. On the other hand, Hurwitz and Meyer (38) reported a more rapid rise in globulin after intraperitoneal injections of bacterial antigens.

The observation that there was some phagocytosis of the dye-protein by fibroblasts between the milk spots of the omentum is important in connection with the subject of sensitization. All of the studies made with carmine and the benzidine dyes (15-19) demonstrated that fibroblasts phagocytize particulate matter as well as macrophages but always in less amount. Experiments with the method of tissue culture have demonstrated that fibroblasts can be sensitized. In 1932 Rich and Lewis (52) showed that in tissues from the spleen of tuberculous guinea pigs the fibro-

blasts were sensitive to tuberculo-protein, although always to a less degree than the neutrophiles and macrophages. This finding was found to be true by Moen and Swift (53).

These studies serve to explain the function of the special form of circulation discovered by Knisely (see Fig. 2 in reference 54) as characteristic of the spleen. He showed, by watching the circulation in the living organ, that the sinusoids frequently collapse through a valve-like action at either end, which traps the red cells and forces a flooding of the plasma from the vessel into the pulp spaces. In the experiments carried out in this laboratory the plasma thus flooded out of the vessels carried free antigen and neutrophilic leucocytes loaded with antigen to surround the monocytes and the macrophages of the splenic pulp. This special vascular mechanism may, therefore, be explained by stating that it serves to give the phagocytic mononuclear cells of this organ time to act.

In all of the experiments with the dye-protein carried out in this laboratory there has been evidence that the neutrophilic leucocytes play a rôle in bringing this antigen into the macrophages. Of course many of the macrophages took up the dye-protein without the aid of the neutrophiles and in their normal function the cells of the spleen are constantly phagocytizing the old neutrophiles; but the presence of the dye-protein in so many of these phagocytized neutrophiles in the spleen, as well as the phagocytosis of neutrophiles containing dye-protein by the Kupffer cells, indicates that the neutrophiles helped to bring this antigen into the reticulo-endothelial cells. The present data thus provide an example of a functional reaction of both neutrophiles and monocytes within the blood stream, whereas it is true that they function for the most part in the tissues, using the blood stream for transport.

These experiments throw some light on the importance of dosage and spacing of the injections of antigens as developed in the experience of immunologists. The cells require time for two processes, namely, the preparation of the antigen for introduction into the cytoplasm and the synthesis of the new globulins. The element of time is therefore of prime importance. The final injection of antigen in rabbit H-670 served to engorge only the Kupffer cells along the portal border of the lobule. A study of the sections of the three rabbits, H-668, 669, and 670, suggests that four or five injections would be required to fill a majority of the Kupffer cells throughout the lobules to the edge of the central vein. Massive doses, on the other hand, such as were given to rabbit R 2503, served to load the cells with much larger aggregates of the dye-protein which, presumably, could not be taken up by the cell as easily as the smaller particles. Thus these data suggest a

justification for the practice of small, divided doses, in that several daily injections of moderate amounts of antigen serve to bring into action a sufficiently large number of phagocytic cells to assure an effective production of antibodies. Studied from 6 to 24 hours after such a series of injections, the cells were found to be filled with antigen in relatively unchanged state and there were no antibodies in the serum. An interval of from 4 to 7 days allows for the production of antibodies and reveals the cells with only a small amount of residual visible antigen. As an illustration of the significance of the time element in these reactions, it is interesting to compare rabbit R 2817 with R 2808 and R 2433. All three animals received the same amount of the dye-protein, namely, 3 mg., but R 2817, which had three daily injections and was studied 24 hours later, had much antigen in the cells and no detectable antibodies, while the other two, which had received spaced injections and were studied 4 days after the last injection, had minimal residual dye in the cells and antibodies in the serum. However, animals vary somewhat in the speed with which they deal with an antigen, for the two rabbits (H-668 and H-669) which had received the same amount of antigen varied in the amount of residual visible antigen in their cells in inverse proportion to the amount of antibodies in the serum.

CONCLUSIONS

1. The use of an antigen which can be seen within cells demonstrates that one may stimulate the phagocytic cells either of the liver and spleen or of the tissues and lymph nodes to produce antibodies.
2. The appearance of antibodies in the serum correlates with the time when the dye-protein is no longer visible within the cells and with the phenomenon of a partial shedding of their surface films.
3. It is thus inferred that the cells of the reticulo-endothelial system normally produce globulin and that antibody globulin represents the synthesis of a new kind of protein under the influence of an antigen.
4. An antigen is a substance which can specifically modify the synthesis of the cytoplasm of the cells of the reticulo-endothelial system.

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EXPLANATION OF PLATE 7

FIG. 1. Photograph of macrophages filled with the alum-precipitated dye-protein (red in color) from a milk spot in a film of omentum of rabbit R 5109, 24 hours after a final injection of 2 mg. of dye-protein intraperitoneally (see Table I). Stained in hematoxylin only so the red granules are due to the material injected. Each of the two macrophages contains one large, round, red globule (lower border of the upper cell and upper right border of the lower cell) which is interpreted as dye separated from the dye-protein and segregated into a single vacuole. The small blue dots in the lower macrophage are remnants of the nucleus (probably nucleic acid) of a neutrophile which had been phagocytized previously. The large, pale nucleus in the center of the figure belongs to a serosal cell. $\times 1,000$.

FIG. 2. Monocyte and Kupffer cell containing dye-protein (red in color) near the portal border of a lobule of the liver from rabbit H-670, 6 hours after a final injection of 8 mg. of the alum-precipitated dye-protein intravenously (see Table I). The long Kupffer cell contains much dye-protein, an unstained neutrophile, and a fragment of a red cell. Near this fragment is a round blue dot from the nucleus (nucleic acid) of a phagocytized cell, probably a neutrophile. Stained in hematoxylin only. $\times 1,000$.

FIG. 3. Kupffer cells containing dye-protein (red in color) near the portal border of the lobule of the liver in the same rabbit as that shown in Fig. 2. These cells show the folds of the surface films of the Kupffer cells which attach themselves to the opposite wall and keep the surface films stretched across the lumen of the vessel for their function of phagocytosis. Stained in hematoxylin only. $\times 1,000$.

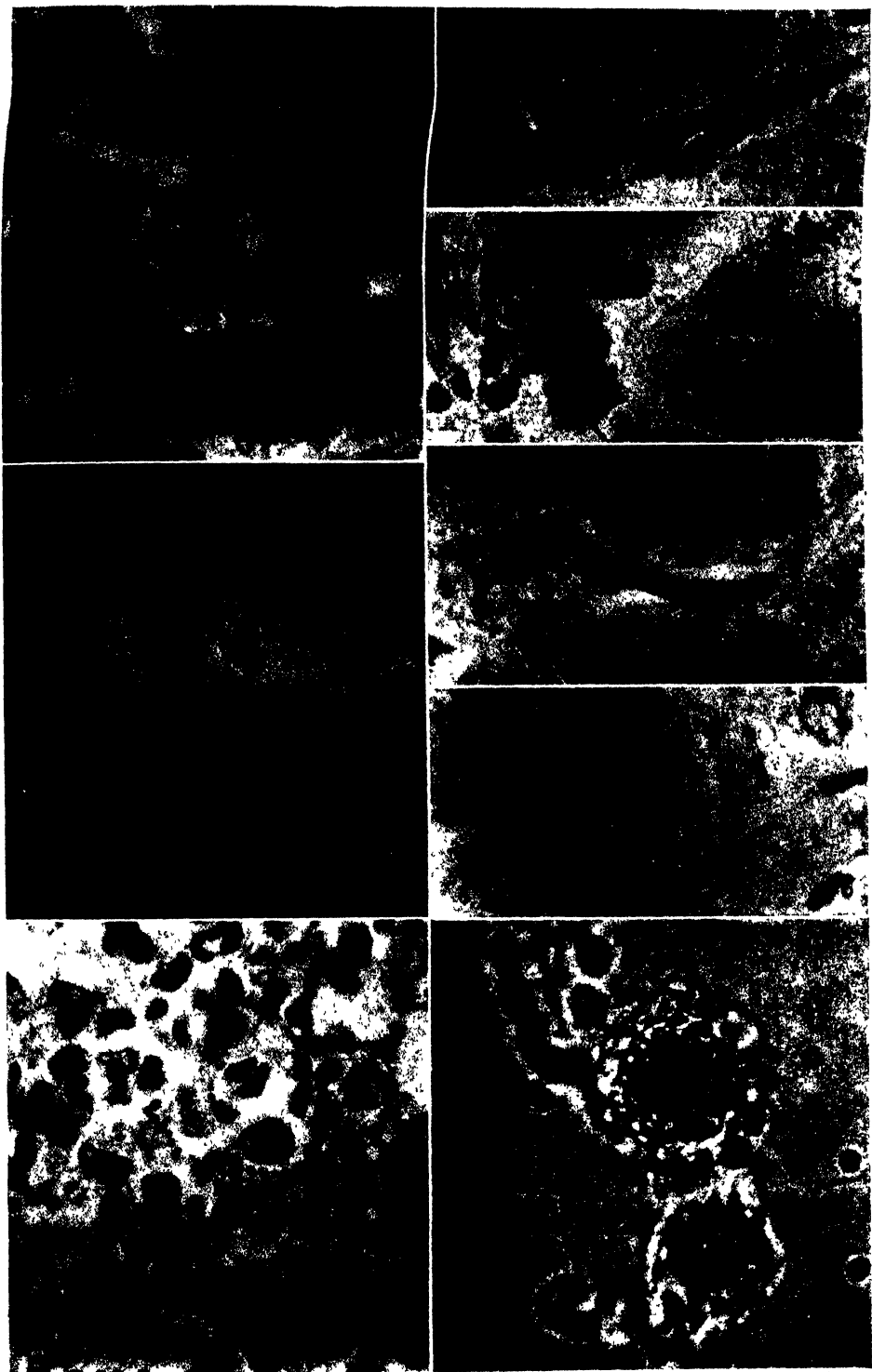
FIG. 4. Multinucleated Kupffer cell containing dye-protein (red in color), not far from the portal border of a lobule of the liver of the same rabbit as shown in Figs. 2 and 3. Stained in hematoxylin only. $\times 1,000$.

FIG. 5. Kupffer cell from the liver of the same rabbit as shown in Figs. 2, 3, and 4, to demonstrate two phagocytized neutrophiles which themselves contain dye-protein, red in color. Stained in hematoxylin only. $\times 1,000$.

FIG. 6. Two Kupffer cells, showing the maximum amount of dye-protein (red in color) found in any one cell in the liver of rabbit H-669, 7 days after a final injection of 8 mg. of the alum-precipitated dye-protein, when there was a relatively high antibody titre in the serum (see Table I). Stained in hematoxylin only. $\times 1,000$.

FIG. 7. Section of the spleen showing a pulp cord from the same rabbit as in Figs. 2, 3, 4, and 5. It contains monocytes (center), macrophages, and a neutrophile (left border) filled with dye-protein, red in color. A large macrophage just below the central monocyte and another in the lower left corner of the photograph show both the red dye-protein and much nuclear debris of phagocytized neutrophiles. Stained in hematoxylin only. $\times 1,000$.

FIG. 8. Two macrophages from the peritoneal exudate of rabbit R 2433, 4 days after an injection of 1 mg. of the alum-precipitated dye-protein (see Table I). These cells were stained with neutral red and were photographed while living. Each one shows a long process of the surface film of the cell about to be shed from the cytoplasm. $\times 1,800$.



Photographed by Joseph B. Haulenbeek

(Sabin: Cellular reactions to dye-protein)

A MOUSE TEST FOR MEASURING THE IMMUNIZING POTENCY OF ANTIRABIES VACCINES

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Recent knowledge of immunity in central nervous system virus infections indicates a need for a restudy of rabies. Questions are now raised as to whether non-virulent vaccines are capable of inducing an active immunity against subsequent test exposure and whether vaccines administered after exposure are capable of controlling the infection. Moreover, with respect to rabies, statistics fail to show superiority of "live" over "killed" vaccines, nor indeed any advantage gained for the most part in commencing vaccine treatment within 14 days after bite (1). Finally, published experiments on the immunizing potency of antirabies vaccines contain meagre evidence that vaccines will immunize against rabies and this evidence is for the most part inconsistent, non-quantitative, and fails to indicate a superiority of one vaccine over another (2).

The need for a quantitative practical test for determining the immunizing potency of rabies vaccines seemed to be met by our finding that inbred W-Swiss mice were highly susceptible and uniform in their response to rabies virus (3). With these mice methods have been developed for the testing of vaccines and reports made of the results of these tests (4). In the present paper these procedures and results are described in more detail.

W-Swiss Mice as Animals of Choice for Testing Antirabies Vaccines

The W-Swiss mice, selectively bred in our laboratory for susceptibility to central nervous system (C.N.S.) virus infections, have proved especially satisfactory for testing antirabies vaccines for the following reasons.

These Mice Are Highly Susceptible and Relatively Uniform in Their Response to Rabies Virus.—Virus-containing brain tissue diluted 1 to 100 with 10 per cent horse serum plus distilled water and injected intracerebrally in 0.03 cc. amounts into 3 weeks old Swiss mice is passed on serially from the brains of the injected mice when they become prostrate. Such virus from prostrate mice gives reliable titration results in repeated tests, as illustrated in Table I.

It will be noted that with increasing dilutions of virus, 50 per cent or more of tested mice succumb regularly until a critical point is reached beyond which less than 50 per cent succumb. This dilution is regarded as the titration end point and as containing 1 minimum lethal dose (M.L.D.) of virus. Duration of life varies little from an average which depends on the amount of virus administered. Other strains of mice and species of animals have given less uniform results. The susceptibility of the 3 weeks old Swiss mouse is high, as shown in Table I, 0.03 cc. of the 10^{-6} or 10^{-7} dilution proving fatal to the majority of individuals tested. This sensitivity is at least 10 times that of guinea pigs or rabbits injected similarly per gram of body weight. Certain other strains of mice have proved equally susceptible (5) but not equally uniform in their response to the virus.

TABLE I

Intracerebral Titrations of Early Passage Dog 15811 Virus in 3 Weeks Old W-Swiss Mice

Test	Passage	Fate of mice given 0.03 cc. mouse-brain virus intracerebrally in dilutions					Titre
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
1	8	*7, 8, 8	8, 8, 11	9, 14, 15	—	—	0.03 cc. $10^{-6}+$
2	17	8, 8	9, 11	12, 14	S, S	—	0.03 " 10^{-6}
3	22	8, 8, 9	10, 10, 11	9, 12, 14	15, 15, S	—	0.03 " 10^{-7}
4	24	—	—	8, 10, 10	10, S, S,	13, S, S	0.03 " 10^{-7}
5	24	7, 9	9, 10, 10, 11	9, 9, 11, 12	11, 11, 12, 12	14, S, S, S	0.03 " 10^{-7}

* Day of death from rabies following injection.

S = mouse remained well. — = dilution not tested.

A similarly uniform response and high susceptibility are obtained if mouse brain passage virus is titred intramuscularly.

Mouse passage virus is prepared in twofold dilutions and 0.01 cc. injected through a 0.3 cm., No. 26 needle pointed distally into the lower third of the gastrocnemius muscle. At least four mice are injected with each dilution of virus. A series of such titrations is illustrated in Table II. The twofold dilutions used in testing this muscle route of infection give less uniform results than the tenfold dilutions employed in testing by the intracerebral route. Nevertheless, 50 per cent or more mice succumb with fair regularity to increasing dilutions of virus, until a point is reached beyond which less than 50 per cent succumb. Duration of life increases regularly with increase in dilution of virus given. The susceptibility of 3 weeks old Swiss mice to this route of infection is high, 0.01 cc. of virus diluted 1 to 320 to 1 to 2,560 generally proving fatal. Other animal species have thus far

proved so irregular in their response to peripheral injection of rabies virus that no quantitative peripheral test has been developed (2).

Certain variables which modify uniformity, regularity, and end points of titrations require careful control. (a) Strains of virus differ in virulence. (b) Strains of virus increase in virulence with mouse brain passage. (c) The age of the mouse modifies the virulence of the virus markedly. These variables will be discussed in detail in a later report.

These mice contract the classical type of disease after injection of a very small amount of virus, 0.01 cc. of a 1 to 320 dilution, into the gastrocnemius muscle. If passage virus is injected, it first appears in the lumbar cord 3 to 5 days later and passes quickly to the brain where it multiplies rapidly. The first signs of disease usually appear on the 9th to 14th days in the form of flaccid paralysis of the injected limb. Convulsions and prostration

TABLE II

Intramuscular Titrations of Early Passage Dog 15811 Virus in 6 Weeks Old W-Swiss Mice

Test	Passage	Fate of mice given 0.01 cc. of mouse-brain virus intramuscularly in dilutions							Titre
		1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	
1	7	*11, 16, 21	13, 14, S, S	11, 14, S, S	—	—	—	—	0.01 cc. 1:320+
2	11	13, 13, 19, S	—	12, 12, 16, S	—	15, 17, S, S	—	—	0.01 " 1:1,280+
3	14	—	—	13, 16, 22	26, S, S, S	13, S, S, S	S, S, S, S	—	0.01 " 1:640
4	17	—	—	11, 13, S	15, 16, S, S	S, S, S, S	S, S, S, S	—	0.01 " 1:640
5	18	—	11, 12, 14, S	14, 17, 18, S	12, 17, S, S	11, 16, 17, S	14, S, S, S	15, S, S, S	0.01 " 1:2,560
6	19	—	15, 15, 21, S	12, 15, 17, S	14, 14, 19, S	12, 14, 14, S	12, 15, S, S	S, S, S, S	0.01 " 1:2,560
7	23	—	13, 13, 14, 16	10, 13, 16, 21	18, 20, 25, S	11, 13, 20, S	13, S, S, S	—	0.01 " 1:1,280
8	23	10, 10, 16, 18	10, 13, 13, 18	10, 13, 13, 15	13, 18, S, S	13, 13, 16, S	13, 13, S, S	—	0.01 " 1:2,560+

* Day of death from rabies following injection.

S = mouse remained well. — = dilution not tested.

follow after 24 hours, terminated usually by death in a varying period of time. An occasional mouse survives. At autopsy lesions are generally limited to the posterior root ganglia, spinal cord, and brain and consist of the characteristic perivascular cuffs of round cells plus an occasional focus of similar cells near a necrotic nerve cell. A few nerve cells in Ammon's horn, posterior ganglia, and cord are usually affected. If street virus is injected, the incubation period and duration of disease are longer. Some of the animals become vicious before paralysis sets in. At autopsy Negri bodies are found in the cells of the lumbar cord but most frequently in the region of Ammon's horn.

The response of 5 weeks old mice to 12th passage virus from dog 15811 is shown in Table III. Depending upon the amount of virus injected, paralysis appeared on the 9th to 17th day and terminated with convulsions

Table IV shows the results of such a test. Batches of 30 day old mice received an intraperitoneal injection of 0.5 cc. of 175th passage mouse brain virus from dog R-1 in dilutions of 1:100, 1:1,000, 1:10,000, and 1:100,000 respectively. A virulence titration run at the same time indicated that the vaccinated mice had received 160,000, 16,000, 1,600, and 160 lethal doses respectively. 3 weeks later their immunity was tested by injecting them intracerebrally with the same strain of virus in dilutions of 1:1,000 through 1:10,000,000. The controls succumbed through the 1:1,000,000 dilution, while the mice vaccinated with 160,000 doses survived 1,000 intracerebral lethal doses; the mice vaccinated with 16,000 doses survived 10 to 100 lethal doses, those vaccinated with 1,600 doses possibly would have survived one lethal dose, and finally those vaccinated with 160 doses were not immunized.

TABLE IV

Immunity of Mice Following Vaccination with Virulent 175th Passage Dog Virus R-1

Vaccine given	No. of intra-cerebral lethal doses in vaccine	Fate of mice given 0.03 cc. of test virus intracerebrally in dilutions						Amount of immunity in intra-cerebral lethal doses
		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
None	None	—	—	—	—	*7, 9, S	9, 11, 12, 12, 13	S, S, S, S, S
0.5 cc. 1:100	160,000	S, S	S, S, S	13, S, S	S, S, S, S	—	—	1,000
0.5 " 1:1,000	16,000	7, S, S	13, S, S, S	13, S, S, S	20, S, S, S	—	—	10-100
0.5 " 1:10,000	1,600	—	7, 7, S, S	7, S, S, S	8, 9, S, S	—	—	1+
0.5 " 1:100,000	160	7, 13, 21	12, 12, 13	11, 12, 13, 21	11, 13, 17, 21	—	—	0

* Day of death from rabies following injection.

S = mouse remained well. — = dilution not tested.

It will be noted besides that the vaccinated mice, unlike the non-vaccinated controls, occasionally showed individual irregularities in response.

This active immunity is influenced by a number of factors which must be carefully controlled. (a) Different strains of virus differ in their immunizing capacity. (b) Different strains of virus differ in their tendency to induce rabies following intraperitoneal injection. (c) The susceptibility of the mice to intraperitoneal injection and their subsequent capacity to be immunized are markedly affected by age. These factors will be considered in detail in a forthcoming paper.

For the purpose of this paper it is sufficient to note that under the conditions described above, immunity can be demonstrated in 7 days and for at least 9 months. Virus given subcutaneously as a vaccine is more active in inducing rabies and less effective in immunizing the mice. Neutralizing antibodies in the serum appear in the intraperitoneally vaccinated mice at

the same time as does immunity. In subcutaneously vaccinated mice, they usually are not demonstrable.

W-Swiss mice fail to react to vaccination commenced subsequent to a test exposure to the virus. Despite the use of a peripheral route of inoculation with a minimum infecting dose of virus, a method of inoculation that is followed by long incubation periods and a mortality rate of less than 100 per cent (Table III), vaccines of all sorts have proved ineffective in our hands in altering the mortality rates or the course of infection from those in unvaccinated mice.

Development of Swiss Mouse Immunity Test

Having learned that W-Swiss mice are highly susceptible and uniform in response to rabies virus and that they can be readily immunized against subsequent, though not prior test exposure to the virus, we set about developing a simple quantitative test for measuring the immunizing potency of a given vaccine against subsequent measured doses of virulent virus.

The first problem had to do with standardizing the size and number of doses of vaccine for injection into the mice: Since most of the commercial vaccines in this country, whether designed for the treatment of man or prophylaxis of animals, have been rendered non-virulent through treatment with phenol or chloroform, they received first consideration. Undiluted vaccine was poorly tolerated by the mice, whether given intraperitoneally as a vaccine or intracerebrally as a test for virulence. Apparently this poor tolerance was due to the inactivating agent. Consequently, each preparation was diluted 1 to 10, regardless of the concentration of brain emulsion or of inactivating agent. This dilution of vaccine proved entirely harmless. The dose of vaccine prescribed for man or dogs obviously could not be employed for mice. $\frac{1}{8}$ th of the prescribed dose, however, was readily tolerated by the mice. Hence a standard practice was adopted of diluting each vaccine 1 to 10 and injecting $\frac{1}{8}$ th of the stated volume, making a total dilution of 1:80. In the case of the vaccines for treatment of man put up in 2 cc. doses, $\frac{1}{4}$ cc. of a 1 to 10 dilution was given; when put up in $\frac{1}{2}$ cc. doses, 0.06 cc. In the case of the vaccines for animal prophylaxis put up in a single 5 cc. dose, 0.6 cc. was given. This diluted dose for a 15 gm. mouse is still approximately 5 times that prescribed for a 10 kilo child or animal per gram of body weight and is therefore regarded as an ample amount for testing.

The intraperitoneal route of injecting the vaccine was chosen on the basis of tests with virulent virus mentioned above. The difference in effectiveness of inactive vaccines given by intraperitoneal and subcutaneous routes is shown later in Experiment 1. A single injection was given if a canine vaccine was being tested. In the case of vaccines for man, six daily doses proved somewhat less effective in mice than fourteen, but three usually sufficed to demonstrate whether the vaccine was antigenic. The difference in effectiveness of a different number of doses is also illustrated in Experiment 1 described below. The time of testing immunity was chosen at 3 weeks after the first dose of vaccine. At this point experience showed immunity to be at a maximum. The test dose was given intracerebrally in the early tests in dilutions containing 1, 10, 100, and

1,000 M.L.D., as shown in Table I. Later, when a study of canine vaccines was made to demonstrate the presence of even a minute amount of immunizing potency, the more sensitive gastrocnemius muscle route was employed in 2, 4, 8, 16, and 32 M.L.D. doses, as shown in Table II. The tested animals, including unvaccinated controls which were of the same age as the vaccinated animals and which were set apart at the outset of the experiment, were observed for 60 days for signs of rabies and then discarded.

To determine whether or not a vaccine as marketed contained virulent virus, it was diluted 1 to 10 and injected intracerebrally in 0.03 cc. amounts into at least five 2 weeks old Swiss mice.

Briefly, the mouse test for measuring the immunizing potency of any antirabies vaccine is carried out as follows: (a) Dilute the vaccine tenfold. (b) Segregate sixteen 3 weeks old Swiss mice for vaccination and sixteen of the same age as controls. Provide at least five additional 2 weeks old mice for the virulence test. (c) Inject the 2 weeks old mice intracerebrally with 0.03 cc. of the diluted vaccine to determine the presence of virulent virus. (d) If the vaccine is designed for the treatment of man, inject sixteen mice with $\frac{1}{8}$ th the stated dose of diluted vaccine intraperitoneally daily for 3 or 6 days. If the vaccine is for canine prophylaxis, inject a single dose of $\frac{1}{8}$ th the stated amount. (e) 3 weeks after the first injection, test vaccinated mice plus controls with 2, 4, 8, and 16 intramuscular lethal doses of virulent virus respectively (or 1, 10, and 100 intracerebral lethal doses).

Details for maintaining titre of the test virus plus additional precautions necessary for consistent results are given above.

Results of Mouse Tests on Commercial Vaccines

Virulent Vaccines for Treatment of Man.—A few vaccines prepared according to the dilution methods of Högyes or Harris were tested with positive results. Upon intracerebral injection they invariably proved virulent but no attempt was made to titrate the amount of virulent virus in the various samples. Injected intraperitoneally as a vaccine, they proved harmless yet immunized the mice within 10 days to 100 intracerebral lethal doses of test virus. The immunity persisted at least 9 months.

Non-Virulent Vaccines for Treatment of Man.—Thirty-two phenolized (Semple) and one chloroformized (Kelser) vaccine for treatment of man obtained from nine different manufacturers have been tested with results shown in Table V. All proved non-virulent. The phenolized products of seven manufacturers have not immunized mice consistently to any significant degree, according to tests on two to four different lots of each. The phenolized vaccine from another manufacturer (No. II), however, has induced a high grade of immunity in mice, as evidenced by the results of tests

of twelve separate preparations. Finally, a chloroformized vaccine from firm No. I also immunized mice readily.

The active phenolized product from firm No. II has been studied in detail with results illustrated in the following protocol.

Experiment 1.—No. II vaccine, a 4 per cent phenolized preparation put up in 14 doses of 2 cc. each, was tested for virulence by diluting it 1 to 10 and injecting 0.03 cc. intracerebrally into 10 mice. It was then given as a vaccine to batches of 75 mice in the following manner. Group A received 1 dose of 0.25 cc. of vaccine diluted 1 to 10 intraperitoneally. Group B received the same dose intraperitoneally for 6 days; group C the same dose intraperitoneally every other day for 6 days; group D 1 dose daily intra-

TABLE V

Results of Mouse Potency Tests of Commercial Non-Virulent Vaccines Designed for Human Treatment

Manufacturer's number	Type of vaccine	Number of preparations tested	Amount of immunity in lethal doses
I	Chloroformized	1	10
II	Phenolized	12	0, 1, 10, 10, 10, 10, 10, 10, 100, 10, 100, 10
III	"	4	1, 0, 0, 0
IV	"	2	0, 0
V	"	3	0, 0, 0
VI	"	3	0, 1, 0
VII	"	3	1, 1, 0
VIII	"	3	1, 1, 1
IX	"	2	0, 0
Total: 9		33	Samples positive from 2 of 9 companies

peritoneally for 14 days. Group E received the fourteen daily doses subcutaneously, and group F received no vaccine. The vaccinated mice were then tested for immunity and for the presence of serum-neutralizing antibodies, 8, 16, 28, 78, 136, and 387 days after the first injection of vaccine.

The results of this experiment are summarized in Tables VI and VII and Text-figs. 1 and 2. The vaccine proved, first of all, to be non-virulent according to the intracerebral test. It immunized in 8 days mice of groups B, C, and D, which had received multiple doses of vaccine intraperitoneally, against 10 to 100 test intracerebral lethal doses of virus. At 16 and 28 days the results were approximately the same and at 78 and 136 days these intraperitoneally vaccinated groups still showed immunity; but at 387 days, when next tested, they were no longer immune. A single dose given to

TABLE VI

Immunity of Mice Following Vaccination with No. II Phenolized Vaccine

Mouse group	Type of vaccination	Time of test after vaccination	Fate of vaccinated mice given 0.03 cc. of test virus intracerebrally in dilutions				Amount of immunity in intracerebral lethal doses
			10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	
		days					
A	i.p., 1 dose	8	—	*10, 10, 12	12, 13, S	S, S, S	1
B	" 6 doses (1 per day)	"	—	13, S, S	S, S, S	S, S, S	100
C	" 6 doses (1 every other day)†	"	—	S, S, S	11, 15, S	S, S, S	1-10
D	" 14 doses (1 per day)	"	—	13, S, S	S, S, S	S, S, S	100
E	s.c., 14 doses (1 per day)	"	—	8, 11, 12	12, 12, 13	13, S, S	1
F	No vaccination	"	—	8, 8, 8	9, 12, 12	11, 11, S	—
A	i.p., 1 dose	16	—	12, 12, 12	12, 12, 12	11, 15, S	0
B	" 6 doses (1 per day)	"	—	14, 14, S	S, S, S	15, S, S	10
C	" 6 doses (1 every other day)	"	—	28, S, S	20, S, S	S, S, S	10-100
D	" 14 doses (1 per day)	"	—	S, S, S	S, S, S	S, S, S	100
E	s.c., 14 doses (1 per day)	"	—	9, 9, 11	11, 11, S	12, S, S	1
F	No vaccination	"	—	8, 10, 10	10, 12, 12	12, 20, S	—
A	i.p., 1 dose	28	—	9, 9, 9	12, 12, S	14, 16, S	0
B	" 6 doses (1 per day)	"	11, 21, S	16, S, S	S, S, S	—	10+
C	" 6 doses (1 every other day)	"	14, 15, S	20, S, S	S, S, S	—	10+
D	" 14 doses (1 per day)	"	S, S, S	S, S, S	S, S, S	—	100+
E	s.c., 14 doses (1 per day)	"	—	8, 9, 10	10, 11, 11	11, S, S	0
F	No vaccination	"	—	8, 9, 9	12, 14, 14	14, S, S	—
A	i.p., 1 dose	78	—	8, 8, 11, 17	10, 11, 17, S	11, S, S, S	0
B	" 6 doses (1 per day)	"	—	10, S, S, S	13, S, S, S	S, S, S, S	1
C	" 6 doses (1 every other day)	"	—	13, S, S, S	S, S, S, S	S, S, S, S	10
D	" 14 doses (1 per day)	"	—	13, 13, S, S	S, S, S, S	13, S, S, S	1
E	s.c., 14 doses (1 per day)	"	—	10, 10, 10, 10	10, 11, 12, S	S, S, S, S	0
F	No vaccination	"	—	10, 10, 10, 10	9, 10, 10, 16	S, S, S, S	—
A	i.p., 1 dose	136	—	8, 9, 9, 12	12, 13, 13, 13	—	<10
B	" 6 doses (1 per day)	"	—	10, 10, 14, S	13, S, S, S	—	10
C	" 6 doses (1 every other day)	"	—	9, S, S, S	18, 21, S, S	—	10
D	" 14 doses (1 per day)	"	—	S, S, S, S	15, S, S, S	—	100+
E	s.c., 14 doses (1 per day)	"	—	10, 10, 11, 13	12, 13, S, S	—	<10
F	No vaccination	"	—	8, 9, 12, 12	12, 13, 13, 14	12, 12, S, S	—
A	i.p., 1 dose	387	—	8, 8, 8, 8	8, 9, 10, 10	10, 11, 16, S	0
B	" 6 doses (1 per day)	"	—	9, 10, 11, 11	12, 13, 17, S	—	0
C	" 6 doses (1 every other day)	"	—	9, 10	9, 12	—	0
D	" 14 doses (1 per day)	"	—	8, 8, 9, 10	9, 9, 9, 11	—	0
E	s.c., 14 doses (1 per day)	"	—	8, 9, 10, 11	9, 9, 9, 10	12, 13	0
F	No vaccination	"	—	6, 7, 8	9, 9, 10	—	0

* Day of death of mouse following injection.

† Only four of total six injections received at time of 8 day test.

S = mouse remained well 30 days. — = dilution not tested.

group A intraperitoneally and 14 doses to group E subcutaneously failed to immunize.

The results of tests for resistance were paralleled by those for neutralizing antibodies except that group A mice, which had received the single intra-peritoneal dose and showed no immunity on inoculation, did show circulating antibodies in every case.

Many preparations of this particular phenolized vaccine (No. II) have been tested. At no time has it been found to contain virulent virus and

TABLE VII

Neutralizing Antibodies in Sera of Mice Vaccinated with No. II Phenolized Vaccine

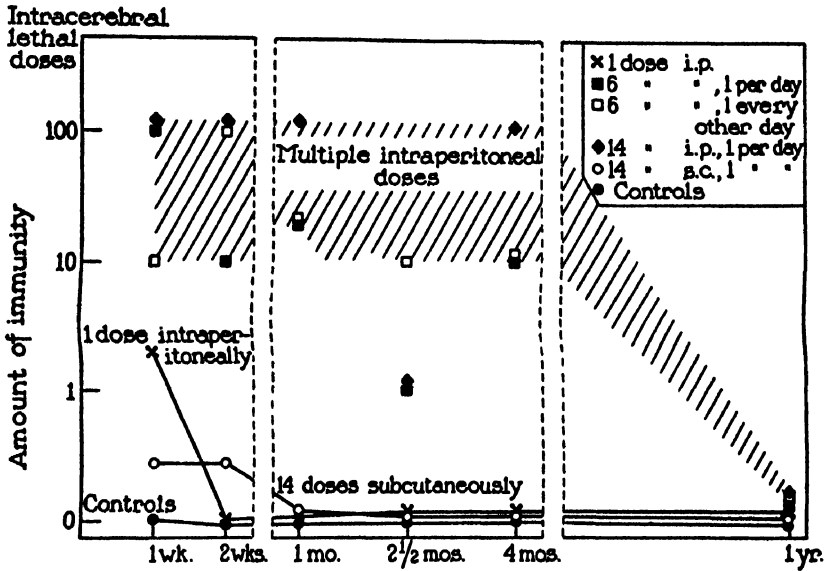
Source of sera		Time of test after vaccination	Fate of mice given 0.03 cc. of serum plus virus in dilutions				Amount of protection in intracerebral lethal doses
Mouse group	Type of vaccination		10 ⁻²	10 ⁻⁴	10 ⁻⁶	10 ⁻⁸	
		days					
A	i.p., 1 dose	8	—	—	S, S, S, S	S, S, S, S	100
B	" 6 doses (1 per day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
C	" 6 doses (1 every other day)†	"	—	—	S, S, S, S	S, S, S, S	"
D	" 14 doses (1 per day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
E	s.c., 14 doses (1 per day)	"	—	*8, 9, 10, 10	10, 10, 12, 12	13, S, S, S	1
F	No vaccination	"	—	8, 8, 8, 10	10, 11, 11, 14	14, 14, 15, S	—
A	i.p., 1 dose	15	—	S, S, S, S	S, S, S, S	S, S, S, S	100+
B	" 6 doses (1 per day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
C	" 6 doses (1 every other day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
D	" 14 doses (1 per day)	"	—	S, S, S, S	S, S, S, S	S, S, S, S	"
E	s.c., 14 doses (1 per day)	"	—	9, 10, 10, 11	10, 11, 12, 12	12, 13, S, S	0
F	No vaccination	"	—	9, 10, 10, 10	11, 12, 13, 13	13, 13, S, S	—
A	i.p., 1 dose	28	11, 12, 12, S	S, S, S, S	S, S, S, S	—	10
B	" 6 doses (1 per day)	"	S, S, S, S	S, S, S, S	S, S, S, S	—	100+
C	" 6 doses (1 every other day)	"	12, S, S, S	S, S, S, S	S, S, S, S	—	"
D	" 14 doses (1 per day)	"	S, S, S, S	S, S, S, S	S, S, S, S	—	"
E	s.c., 14 doses (1 per day)	"	—	9, 10, 10, 10	10, 12, 13, 13	13, S, S, S	0
F	No vaccination	"	—	9, 9, 11, 11	10, 11, 13, 13	13, S, S, S	—
A	i.p., 1 dose	136	9, 9, 9, 9	9, 10, 11, 12	11, 11, 11, 13	—	0
B	" 6 doses (1 per day)	"	S, S, S, S	S, S, S, S	S, S, S, S	—	1,000
C	" 6 doses (1 every other day)	"	S, S, S, S	S, S, S, S	S, S, S, S	—	"
D	" 14 doses (1 per day)	"	12, S, S, S	13, 14, S, S	S, S, S, S	—	100
E	s.c., 14 doses (1 per day)	"	—	8, 8, 8, 9	10, 10, 11, 11	12, 13, 13, 13	0
F	No vaccination	"	—	9, 9, 9, 10	11, 11, 13, 13	11, 15, S, S	—

* Day of death of mouse following injection.

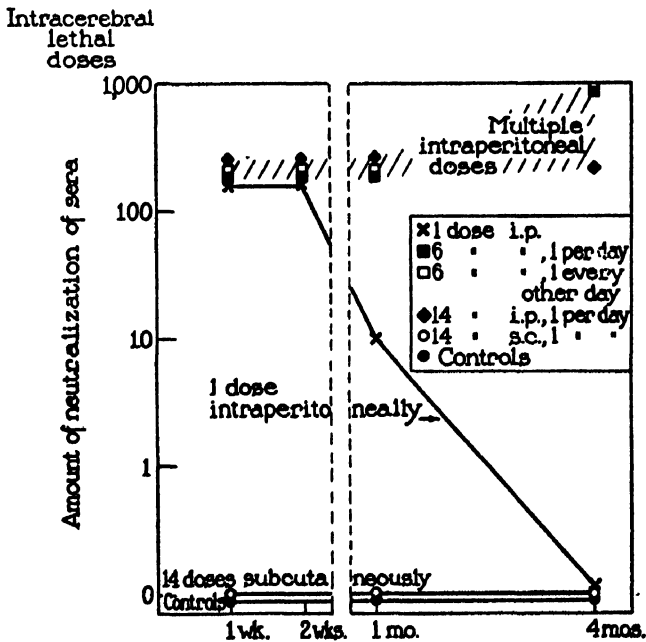
† Only four of total six injections received at time of 8 day test.

S = mouse remained well 30 days. — = dilution not tested.

yet it has never failed on test to induce neutralizing antibodies in human beings or in experimental animals and has rarely failed to render experimental animals immune. The immunity corresponds to that produced with virulent virus. It appears on or about the 8th day and endures approximately a year. Six doses give somewhat less immunity than the full



TEXT-FIG. 1. Immunity of mice to rabies following injection with a virulent phenolized vaccine.



TEXT-FIG. 2. Neutralizing antibodies in blood serum of mice following injection with avirulent phenolized antirabies vaccine.

fourteen doses. The material must be given intraperitoneally, not subcutaneously, for an appreciable or consistent effect. Antibodies appear following a single intraperitoneal injection, although this amount is insufficient to render the animal immune to an intracerebral test dose. The minimum quantity required for immunization of mice is at least 5 times that prescribed for human beings per gram of body weight.

The vaccines described above which failed to immunize mice against a subsequent intracerebral test inoculation likewise failed or showed very little tendency to immunize against the peripheral test inoculation. The two vaccines which successfully immunized against the intracerebral were likewise effective against the peripheral inoculation. The following

TABLE VIII

Immunizing Potency of No. I Chloroform Vaccine in Mice against a Subsequent Intramuscular Test Infection

Mouse group	Fate of mice inoculated intramuscularly with virus in dilutions					Amount of immunity in intramuscular lethal doses
	1:20	1:40	1:80	1:160	1:320	
Non-vaccinated controls.	*7, 8, 8, 13, 16	9, 9, 10, 10, 13	8, 9, 9, 9, 13	9, 10, 10, S, S	9, 11, S, S, S	8+
Vaccinated	S, S, S, S, S	S, S, S, S, S	S, S, S, S, S	S, S, S, S, S	S, S, S, S, S	

* Day of death of mouse following test injection.

S = remained well 30 days.

protocol illustrates the immunity conferred by non-virulent No. I chloroformized vaccine against a subsequent intramuscular injection of virus.

Experiment 2.—Twenty mice received 0.06 cc. of No. I chloroformized vaccine, diluted 1:10, daily for 14 days. Twenty-five similar mice were set aside as controls. 3 weeks later both groups were tested intramuscularly. 0.01 cc. of virus in dilutions of 1:20 through 1:160 was given to each of five vaccinated mice respectively. The same dilutions plus 1:320 were likewise given to the non-vaccinated controls.

The results in Table VIII show that 100 per cent of non-vaccinated mice succumbed to the test virus through the 1:80 dilution, 60 per cent in the 1:160, and 20 per cent in the 1:320 dilution. The end point, therefore, is taken as 1:160. All vaccinated mice were immune to the 1:160, 1:80, 1:40, and 1:20 dilutions and hence to at least eight intramuscular lethal doses.

The next experiment illustrates the effect of decreasing dosage of vaccine on the amount of immunity obtained.

Experiment 3.—Batches of sixteen 3 weeks old mice were vaccinated in the following manner. Batch A received six daily intraperitoneal doses of 0.25 cc. and batch B 0.05 cc. of No. II phenolized vaccine, diluted 1 to 10. Batch C received six daily intraperitoneal doses of 0.06 cc. and batch D 0.012 cc. of No. I chloroformized vaccine diluted 1 to 10. Batch E was reserved as controls. To detect the presence of virulent virus, each diluted vaccine was injected intracerebrally in 2 weeks old mice. 3 weeks later the vaccinated and control mice were tested for immunity against the intramuscular injection of virus.

The virulence test showed no evidence of virulent virus in the vaccines and the immunity tests gave results shown in Table IX. No. II vaccine given in six 0.25 cc. doses, which is a total amount equivalent to about 5

TABLE IX
*Immunizing Effects on Mice of Antirabies Vaccines
Phenolized and Chloroformized Vaccines for Treatment of Man*

Mouse group	Fate of mice inoculated intramuscularly with 0.01 cc. of virus in dilutions					Amount of immunity in intramuscular lethal doses
	1:80	1:160	1:320	1:640	1:1,280	
A. Phenolized No. II 0.25 cc.	*10, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	4
B. Phenolized No. II 0.05 cc.	16, 21, S, S	11, 25, S, S	S, S, S, S	9, S, S, S	—	1
C. Chloroformized No. I 0.06 cc.	S, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	4
D. Chloroformized No. I 0.012 cc.	10, 15, S, S	24, S, S, S	11, S, S, S	S, S, S, S	—	1
E. Controls	9, 10, 11, 16	—	9, 12, 19, 22	—	S, S, S, S	—

* Day of death from rabies following injection.

S = mouse remained well. — = dilution not tested.

times that for a 10 kilo child per gram of body weight, protected the mice against at least four lethal intramuscular doses, whereas when given in $\frac{1}{16}$ th that amount, which more nearly approximates the relative amount given to a child, the result was negative. The same proved true for the chloroformized No. 1 vaccine. Apparently the minimum total dosage sufficient to induce immunity in mice is in the neighborhood of 5 times that regularly prescribed for human beings per gm. of body weight.

Non-Virulent Vaccines for Canine Prophylaxis.—According to United States Government regulations, all vaccines for animal antirabies prophylaxis must be non-virulent. The questions at issue in this study were therefore twofold: First, are the products now on the market non-virulent

according to the mouse test; and second, do they immunize mice against at least two lethal doses of test virus given under controlled and yet as nearly natural conditions as possible?

The following results have been obtained on tests of five or more lots of vaccine from each of ten commercial firms, 50 preparations in all:—

None has contained virulent virus. None has immunized mice against a subsequent test intracerebral injection of one lethal dose. When tested by the intramuscular method (Table X), twenty-seven phenolized prepara-

TABLE X
Results of Potency Tests (Intramuscular) of Canine Vaccines

Manufacturer's number	Type of preparation	Number of preparations tested	Amount of immunity in lethal doses
1	Chloroformized	7	8, 8, 2, 4, 4, 8, 8
3	"	3	4, 16, 8
2	"	4	4, 0, 2, 2
3	Phenolized	4	0, 4, 2, 2
4	"	4	0, 2, 0, 0
5	"	4	0, 0, 0, 0
6	"	4	0, 1, 0, 0
7	"	4	0, 0, 2, 2
8	"	3	0, 0, 0
9	"	4	2, 4, 0, 0
Total: 9 manufacturers		41 preparations	10 of 10 chloroformized vaccines from 2 manufacturers, positive; 3 of 4 chloroformized vaccines from 1, and 27 phenolized vaccines from 7 manufacturers, negative

tions from seven manufacturers for the most part proved negative. Chloroformized vaccines, on the other hand, especially from manufacturers Nos. 1 and 3, have given results which merit further study.

The following protocols illustrate the intramuscular potency test for canine vaccines and the type of result obtained.

Experiment 4.—Batches of sixteen 3 weeks old Swiss mice were given a single injection of commercial vaccine diluted 1 to 10 in the following manner. Batch A received 0.6 cc. of No. 1, 20 per cent chloroformized vaccine intraperitoneally, and batch B 0.1 cc. of the same dilution. Batch C received 0.6 cc. of No. 1, 33½ per cent chloroformized vaccine intraperitoneally, and batch D 0.6 cc. of the 20 per cent preparation subcutaneously.

Batch E was given 0.6 cc. of No. 2 vaccine intraperitoneally, batch F 0.1 cc. of the same preparation, while batch G received 0.6 cc. subcutaneously. Batch H was reserved without vaccination as controls. At the same time 0.03 cc. of each vaccine diluted 1 to 10 was injected intracerebrally into five mice.

3 weeks later the vaccinated and control mice were tested against an intramuscular injection of a mouse brain passage strain, 0.01 cc. of virus in dilutions of 1:20 to 1:320 being injected into the gastrocnemius muscle.

The chloroformized vaccines proved non-virulent. In the immunity test (Table XI) all dilutions of virus through 1:320 proved fatal to the non-vaccinated mice. Since no further dilutions were tested, 1:320 is taken as the end point, although the titre may have been still higher. The No.

TABLE XI

Immunizing Effect of Canine Antirabies Vaccine on Mice
Comparison of Effects of Subcutaneous and Intraperitoneal Routes of Injecting Vaccine

Mouse group	Route of vaccination	Fate of mice inoculated intramuscularly with virus in dilutions					Amount of immunity in intramuscular lethal doses
		1:20	1:40	1:80	1:160	1:320	
A. No. 1 chloroformized 20% 0.6 cc.	i.p.	*8, 9, 21	9, 10, 25, S	10, S, S, S	S, S, S, S	—	4
B. " " " 0.1 "	"	7, 8, 15, 29	8, 8, 11, S	8, 8, 10, S	8, S, S, S	—	2
C. " " 33½% 0.6 "	"	8, S	9, 9, S	8, 14, 16	S, S, S	—	2
D. " " 20% 0.6 "	s.c.	8, 8, 12	7, 7, 10, S	8, 12, 13, S	9, 9, 10, S	—	0
E. No. 2 " " 0.6 "	i.p.	8, 8, 18	8, 8, 9	8, 9, 9, 13	11, 11, 13, S	—	0
F. " " " 0.1 "	"	7, 8, 8, 8	8, 9, 9, 9	11, 11, 18, S	9, 10, 13, 15	—	0
G. " " " 0.6 "	s.c.	7, 10, 15	8, 9, 9	8, 8, 13	8, 9, 11, 14	—	0
H. No vaccine		—	8, 8, 8	9, 15, 22	10, 10, 11, 15, 8, 10, 22, 23		

* Day of death from rabies following test injection.

S = mouse survived 40 days. — = dilution not tested.

2 vaccine failed to immunize, whether given subcutaneously or intraperitoneally. The No. 1 vaccine likewise failed to immunize when given subcutaneously, whereas intraperitoneally in 0.6 cc. or 0.1 cc. doses it immunized against four and two lethal doses respectively. The dose of 0.6 cc. represents 5 times the dose for 10 kilo dogs per gram of body weight, while 0.1 cc. corresponds to the canine dose.

0.6 cc. of chloroformized vaccine given intraperitoneally appeared to irritate the peritoneum. The mice seemed to be in pain for about an hour, were hyperirritable, and occasionally developed transitory convulsions. The discomfort, although causing loss of appetite for a day or so, seemed relatively harmless.

Experiment 5.—Batches of thirty Swiss mice 3 to 4 weeks of age were given a single intraperitoneal injection of vaccine diluted 1 to 10 in the following manner. Batch A

received 0.6 cc. of No. 3 chloroformized vaccine and batch B 0.1 cc. of the same preparation; batch C 0.6 cc. of No. 3 phenolized vaccine, and batch D 0.1 cc. of the same sample; batch E received 0.6 cc. of No. 2 chloroformized vaccine, batch F 0.1 cc., and batch G 0.6 cc. of No. 7 phenolized vaccine. Batch H remained unvaccinated as con-

TABLE XII
Immunizing Effects of Canine Antirabies Vaccine on Mice
Comparison of Chloroformized and Phenolized Vaccines

Mouse group	Test virus	Fate of mice inoculated intramuscularly with 0.01 cc. virus in dilutions							Amount of immunity in intramuscular lethal doses
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
A. No. 3 chloroformized 0.6 cc.	15811	*20, S, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	—	—	16
B. No. 3 chloroformized 0.1 cc.		9, 15, 20, S	20, S, S, S	14, S, S, S	18, S, S, S	—	—	—	2
C. No. 3 phenolized 0.6 cc.		8, 10, 11, 11	12, 15, S, S	15, 18, S, S	14, S, S, S	—	—	—	2
D. No. 3 phenolized 0.1 cc.		9, 18, 20, 20	17, 18, 18, S	12, 26, S, S	12, 15, S, S	—	—	—	2
E. No. 2 chloroformized 0.6 cc.		10, 11, 12, 20	15, 17, 18, S	15, S, S, S	20, S, S, S	—	—	—	2
F. No. 2 chloroformized 0.1 cc.		12, 15, 19, S	10, 12, 15, S	10, 17, 18, 27	16, 24, S, S	—	—	—	<2
G. No. 7 phenolized 0.6 cc.		10, 16, 20, 20	13, 18, 18, S	14, 23, S, S	S, S, S, S	—	—	—	2
H. No vaccine		10, 10, 12	9, 11, 13, 18	11, 17, 28	13, 14, S, S	10, 15, S, S	—	—	
A. No. 3 chloroformized 0.6 cc.	Sk.	—	9, 17, S, S	S, S, S, S	S, S, S, S	S, S, S, S	—	—	8
B. No. 3 chloroformized 0.1 cc.		—	12, 16, S	S, S, S, S	S, S, S, S	15, S, S, S	—	—	8
C. No. 3 phenolized 0.6 cc.		—	9, 17, S	9, 9, 9, S	11, 11, 15, S	10, S, S, S	—	—	2
D. No. 3 phenolized 0.1 cc.		—	7, 9	9, 9, S	9, 10, S, S	9, 10, 14, S	—	—	0
E. No. 2 chloroformized 0.6 cc.		—	7, 9, 15	9, S, S	29, S, S, S	28, S, S, S	—	—	2
F. No. 2 chloroformized 0.1 cc.		—	7, 9, 10	9, 10, 13	9, 10, 12, S	10, 12, 15, S	—	—	0
G. No. 7 phenolized 0.6 cc.		—	9, 10, 15	10, 10, S	12, 15, S, S	10, S, S, S	—	—	2
H. No vaccine		—	—	9, 9, 10, 11	9, 11, 12, 12	9, 9, 10, 10	9, 15, S, S	S, S, S, S	

* Day of death from rabies following test injection.

S = mouse survived 40 days. — = dilution not tested.

trols. At the same time, 0.03 cc. of each vaccine diluted 1 to 10 was injected intracerebrally into five mice.

3 weeks later the vaccinated and control mice were divided into two equal lots for testing against two strains of rabies virus, one recently isolated from a rabid dog and passed through seven mice, the other isolated from a skunk and passed through 156 mice.

0.01 cc. of virus in dilutions from 1:20 to 1:1,280 was injected into the gastrocnemius muscle.

The results of this experiment are shown in Table XII. All vaccines proved non-virulent. The early passage 15811 test virus was somewhat irregular in its effects but was fatal to at least two of four mice through the 1:320 dilution. 0.01 cc. of 1:320 is taken, therefore, as 1 lethal dose, although it is possible that the titre may have been somewhat higher. None of the vaccines gave more than 2 M.L.D. protection except the No. 3 chloroformized preparation. This vaccine, in the 0.6 cc. dose, which is approximately 5 times that for dogs per gram of body weight, protected the vaccinated mice against sixteen lethal muscle doses. 0.1 cc., the dose corresponding to that for dogs, actually seemed to give some immunity, although if one considers the fatalities in high dilutions as retroactive, the irregularities bring the immunity below the level of significance. The results following injection of similar batches with the 156 mouse passage Sk. strain were more regular and similar to those with the 15811 strain. The virus in non-vaccinated mice was fatal regularly through the 1:320 dilution and to two of four in the 1:640 dilution. This is taken, therefore, as the end point. No vaccine except the No. 2 chloroformized preparation immunized the mice against more than 2 M.L.D. of test virus. This latter vaccine, however, again immunized not only against eight muscle doses, when given in a 0.6 cc. dose, but equally as well in a 0.1 cc. dose.

The chloroformized vaccines given intraperitoneally again proved irritative in contrast to the phenolized preparations which caused no reaction.

Experiment 6.—Batches of fifteen mice, 3 to 4 weeks old, were given a single intraperitoneal injection of vaccine diluted 1 to 10 in the following manner. Batch A received 0.6 cc. of No. 1, 20 per cent chloroformized vaccine and batch B 0.1 cc. of the same preparation. Batch C received 0.6 cc. of No. 1, 33½ per cent chloroformized vaccine, and batch D 0.1 cc. of the same. Batch E was given 0.6 cc. of No. 5 phenolized vaccine, batch F 0.6 cc. of No. 6 vaccine, and batch G 0.6 cc. of No. 8. Batch H was left unvaccinated as controls. At the same time, 0.03 cc. of each vaccine diluted 1 to 10 was injected intracerebrally into five mice.

3 weeks later the unvaccinated and vaccinated mice were given the early passage 15811 strain of rabies virus, precisely as described in Experiment 5.

The results of this test are given in Table XIII. The vaccines proved non-virulent. Again, the early passage strain proved somewhat irregular in its effects but killed 50 per cent or more mice through the 1:1,280 dilution which was regarded as the end point. The phenolized vaccines again proved generally non-effective but the No. 1 chloroformized preparation, whether in 20 per cent or 33½ per cent concentration, immunized the mice

against at least eight intramuscular lethal doses. 0.1 cc. of the 20 per cent vaccine likewise immunized against four lethal doses.

Taken together, the experiments show that no vaccines given subcutaneously, and no phenolized vaccines given either subcutaneously or intraperitoneally immunized mice against more than two intramuscular lethal doses of test virus. Chloroformized vaccines from two manufacturers, however, immunized against four to sixteen doses when given intraperitoneally in amounts 5 to 10 times that advocated for dogs per gram of body

TABLE XIII
Immunising Effects of Canine Antirabies Vaccines
Further Comparison of Chloroformized and Phenolized Vaccines

Mouse group	Fate of mice inoculated intramuscularly with virus in dilutions						Amount of immunity in intramuscular lethal doses
	1:40	1:80	1:160	1:320	1:640	1:1,280	
A. No. 1 chloroformized 20% 0.6 cc.	*13, 14, 23	17, S, S, S	15, S, S, S	18, S, S, S	—	—	8
B. No. 1 chloroformized 20% 0.1 cc.	12, 12, 13	12, 12, 13, 15	12, 13, 15, 16	17, S, S, S	—	—	4
C. No. 1 chloroformized 33½% 0.6 cc.	21, S	16, 20, S, S	14, S, S, S	12, S, S, S	—	—	8
D. No. 1 chloroformized 33½% 0.1 cc.	11, 15, 17	12, 13, 14, S	17, 19, S, S	16, 17, 22, S	—	—	<4
E. No. 5 phenolized 33½% 0.6 cc.	—	12, 13, 17	13, 14, 15, 17	14, 29, S, S	S, S, S, S	—	2
F. No. 6 phenolized 33½% 0.6 cc.	—	13, 16, S	12, 22, S, S	12, 12, 20, S	12, 19, S, S	—	0
G. No. 8 phenolized 33½% 0.6 cc.	—	13, 17, S	12, 16, S, S	12, 30, S, S	13, 17, 31, 2	—	0
H. No vaccine	13, 17, 25	12, 12, 30, S	—	12, 12, 17	—	15, 16, S, S	—

* Day of death from rabies following test injection.

S = mouse survived 40 days. — = dilution not tested.

weight, and occasionally when given in amounts corresponding to the canine dose. The chloroformized vaccines given intraperitoneally in 0.6 cc. doses caused transitory irritative phenomena.

DISCUSSION

The mouse test described in this paper is believed to be reliable and at the same time to reproduce field conditions of exposure adequately. According to the results presented, vaccination with 10,000 intracerebral lethal doses of virus, although potentially dangerous, nevertheless immunizes 3 weeks old mice against four or more intramuscular lethal doses

of test virus. Most commercial phenolized vaccines fail to immunize but Kelser's chloroformized preparations generally give positive results. The original virus-containing brain tissue probably contains about 3.3×10^6 mouse intracerebral lethal doses per cc. before inactivation and 1.1×10^6 doses in the $33\frac{1}{3}$ per cent suspension. Following inactivation with chloroform, about 0.5 cc. of a 1 to 10 dilution, or 55,000 inactivated intracerebral mouse doses, is required to immunize a 3 weeks old mouse against four or more intramuscular test doses. These comparative figures must be regarded, of course, as crude comparative approximations not as definite values.

The commercial vaccine of choice for further study is, according to the mouse test, the Kelser chloroformized vaccine; the dose for mice, 2 to 5 times that now advocated for man or animals; the route, intraperitoneal instead of subcutaneous. This preparation requires 60 to 90 days for complete inactivation and under the above conditions is irritating to the peritoneal cavity.

These findings in mice require rigid checking in other animal species. Such experiments are now in progress in dogs.

CONCLUSIONS

1. A quantitative practical mouse test is described for measuring the immunizing potency of antirabies vaccines.

2. Virulent virus, injected intraperitoneally as a vaccine, immunized mice within 10 days and for a period of at least 9 months. Demonstrable neutralizing antibodies accompanied this immunity. Virus given subcutaneously failed to immunize as effectively. The margin between immunizing and infecting dose of vaccine was small.

3. Commercial vaccines containing virulent virus prepared for the treatment of man gave results similar to those obtained with laboratory virus.

4. Commercial vaccines inactivated with phenol and prepared for the treatment of man in general failed to immunize mice. None contained virulent virus. The phenolized preparation from one commercial firm, however, as also the chloroformized preparation from another, immunized mice consistently when given intraperitoneally in quantities approximating 5 times that advocated per gm. of body weight in man.

5. Commercial canine vaccines inactivated with phenol proved non-virulent and failed to immunize mice.

6. Commercial canine vaccines inactivated with chloroform (Kelser) proved non-virulent but capable of immunizing mice provided a single

intraperitoneal injection of 2 to 5 times that prescribed for dogs per gm. of body weight was given.

7. Chloroformized vaccines proved irritative to the peritoneum of mice.

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RÔLE OF INBORN RESISTANCE FACTORS IN MOUSE POPULATIONS INFECTED WITH BACILLUS ENTERITIDIS

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The experiments described in this paper deal with the rôle of inborn resistance factors of the host in determining the severity of epidemics and with the question of whether inborn resistance or acquired immunity is the more important in determining survivorship.

Technique

Mice used in these experiments were of four sorts.

W-Swiss strain mice were used as carriers in Experiments 1 and 2. These mice have been selectively bred by us for susceptibility to central nervous system virus infections but not to mouse typhoid. Following a *per os* instillation of 5,000,000 *B. enteritidis* mouse typhoid bacilli, 50 to 60 per cent succumb regularly.

White-face strain mice were used as carriers in Experiment 3. These mice are regarded as a genetically pure line. Following a *per os* instillation of 5,000,000 mouse typhoid bacilli, 95 to 100 per cent succumb regularly (1).

Selectively bred susceptible and resistant Rockefeller Institute mice were the actual test animals employed in all experiments. These lines originated from the Rockefeller Institute strain, pen inbred and maintained on a special Steenbock diet, of which 42 per cent succumb to 5,000,000 *B. enteritidis* mouse typhoid organisms given by stomach tube. Following selective inbreeding of this R. I. strain (2), a resistant line was obtained, of which not more than approximately 10 per cent succumb to the standard test dose of *B. enteritidis*. A susceptible line was likewise selected, of which approximately 90 per cent succumb to the test dose. The resistant line also withstands 1,000 times the dose fatal to the susceptible line. We had for testing, therefore, individuals of high inbred resistance to mouse typhoid under standard conditions, nine of ten of which would survive or succumb to infection according to prediction.

These mice were combined in various proportions to make up populations into which mouse typhoid was introduced. A single cage of standard size was used for simplicity, although a crowding factor exerted a definite influence on mortality. Mouse typhoid was introduced by feeding *B. enteritidis* by stomach tube to certain individuals and subsequently adding them to the population and permitting the resulting infection to spread among the constituents "naturally." Cages were cleaned as routine. The modified Steenbock diet was employed (1). Dead mice were autopsied and cultures taken of spleen for identification.

The strain of *B. enteritidis* organisms used in these experiments was obtained origi-

nally from a wild mouse and since that time has been maintained in the laboratory under conditions in which virulence has remained at a constant level (3).

EXPERIMENTS

The first experiment was designed to study the epidemiology of mouse typhoid in a population in which at least 50 per cent of the mice were known to be inherently resistant.

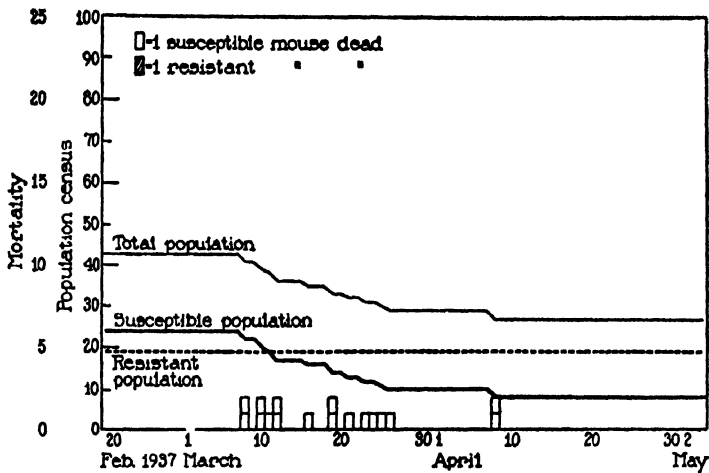
Experiment 1.—Twenty-four Swiss mice with identifying marks were placed in a pen with nineteen mice of the selected resistant strain likewise marked. Ten mice of the selected susceptible line were marked and each given by stomach tube 0.5 cc. of broth containing 5,000,000 *B. enteritidis* mouse typhoid organisms. 24 hours later they were added to the above population. The usual feeding and maintenance routines were instituted and mice prostrate or dead were autopsied as far as possible and cultured for the presence of mouse typhoid organisms.

The experimentally infected mice commenced dying on the 6th and were all dead of mouse typhoid by the 9th day. The Swiss contacts commenced dying on the 18th day and continued thereafter for 32 days (Text-fig. 1). During this period sixteen Swiss mice (66 per cent) died, of which thirteen were autopsied and proved positive for mouse typhoid bacilli. This 66 per cent mortality was in accord with the prediction based on *per os* titrations. We had, of course, no prior knowledge as to which Swiss individuals would succumb and therefore did not know whether the 34 per cent which survived were at the outset inherently resistant or had acquired an immunity through non-fatal infection and had therefore survived. 26 days following the last fatality the eight Swiss survivors were sacrificed and autopsied. Five (62 per cent) showed positive spleen cultures and one serum agglutinins titering 1 to 10.

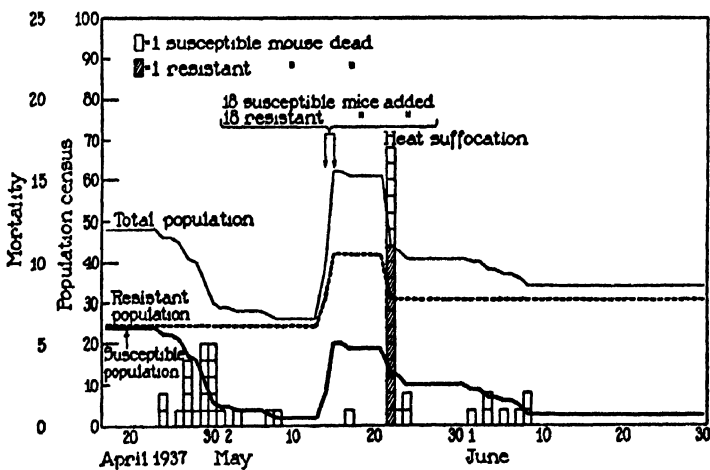
The striking result of this test is that, according to prediction, none of the inherently resistant mice succumbed. At autopsy, however, following completion of the experiment, eight (58 per cent) had positive spleens. The relative proportion of susceptibles and resistants infected was similar, although mortalities were widely different. This relationship has been pointed out before as indicating plainly that so called infectivity *versus* virulence phenomena cannot be regarded as distinctive properties of the parasite (Greenwood, Hill, Topley, and Wilson, 4), but can be shown by epidemiological experiment to be phenomena dependent upon host differences.

The second experiment was planned to study the epidemiology of mouse typhoid in a population in which the inherent resistance of each individual was predictable to within a 10 per cent error.

Experiment 2.—Twenty-four selected susceptible and twenty-four selected resistant mice were given identification marks and placed in a standard pen. 3 days later, sixteen Swiss mice were labeled and each given by stomach tube 0.5 cc. of broth containing 5,000,000 *B. enteritidis* mouse typhoid organisms. 24 hours later they were added to



TEXT-FIG. 1



TEXT-FIG. 2

TEXT-FIGS. 1 and 2. Fate of susceptible and resistant mice exposed to *B. enteritidis* mouse typhoid (Experiments 1 and 2).

the above population. Feeding, maintenance, autopsy, and culture routines were practiced as in Experiment 1.

The experimentally infected Swiss mice commenced dying of mouse typhoid on the 6th day and were all dead save three by the 10th day.

Another succumbed 7 days later, a second after 2 more weeks, and the remaining mouse survived. The susceptible contacts commenced dying on the 8th day (Text-fig. 2). During the next 15 days mortalities were of epidemic proportions, taking all but two. From the spleen of each fatal case, mouse typhoid bacilli were recovered.

The resistant contacts remained well according to prediction during this period in which their susceptible cage mates, in intimate contact, were experiencing an epidemic. When the outbreak subsided they were the sole survivors with the exception of two susceptible contacts plus two injected Swiss mice. It was clear, therefore, in this experiment that the twenty-six survivors of a mouse typhoid epidemic were the individuals (92 per cent) with high inborn resistance at the outset.

The failure of the susceptibles to survive through development of immunity was thought to be due possibly to the large dosage of organisms excreted by the infected Swiss mice. If susceptibles were exposed during the post-epidemic period, when no deaths were occurring and organisms were spread chiefly by the resistant survivors, the ensuing events might be different. Consequently, on the 28th day, six susceptible plus six resistant mice were added and on the 29th day, twelve susceptibles and twelve resistants.

On the 2nd day following, one of the original susceptibles died, leaving only one remaining. 5 days later an accident occurred resulting in heat suffocation of twenty mice in 3 days. Nine of these were original resistants. Only four could be autopsied but no typhoid was found. Two were resistant recruits, likewise negative for mouse typhoid. One was the last remaining original susceptible and one an original Swiss, while eight were susceptible recruits. Only six could be autopsied, but of these four were positive. This experience indicates that the resistants, although resistant to typhoid, were proportionately no more resistant to heat than the susceptibles.

8 to 15 days later a small outbreak occurred among the ten remaining susceptible recruits, fatal to seven. These were all positive for *B. enteritidis* at autopsy. After 21 days without fatalities, the experiment was discontinued.

Of all the susceptibles, only three remained, whereas all of the resistants remained except the eleven destroyed by suffocation. 90 per cent of the surviving population, therefore, was comprised of individuals known at the outset to have high inborn resistance.

Experiments 1 and 2 together show that (a) mortality from mouse typhoid was confined almost exclusively to the mice of the inherently sus-

ceptible line; (b) very few susceptibles survived, thus minimizing the possibility that survivors are immunized susceptibles; (c) all, or nearly all, the mice of the inherently resistant line remained well and hence constituted practically the entire population of survivors; (d) a considerable proportion of the resistant mice which survived had infected spleens, showing that a single strain of mouse typhoid bacilli can be infective but not lethal (virulent) in one strain of mice, and in another, under similar conditions, both infective and lethal; (e) infection was spread by the resistant survivors to the susceptible immigrants.

The next experiment was planned to test further the selective mortality among the susceptibles.

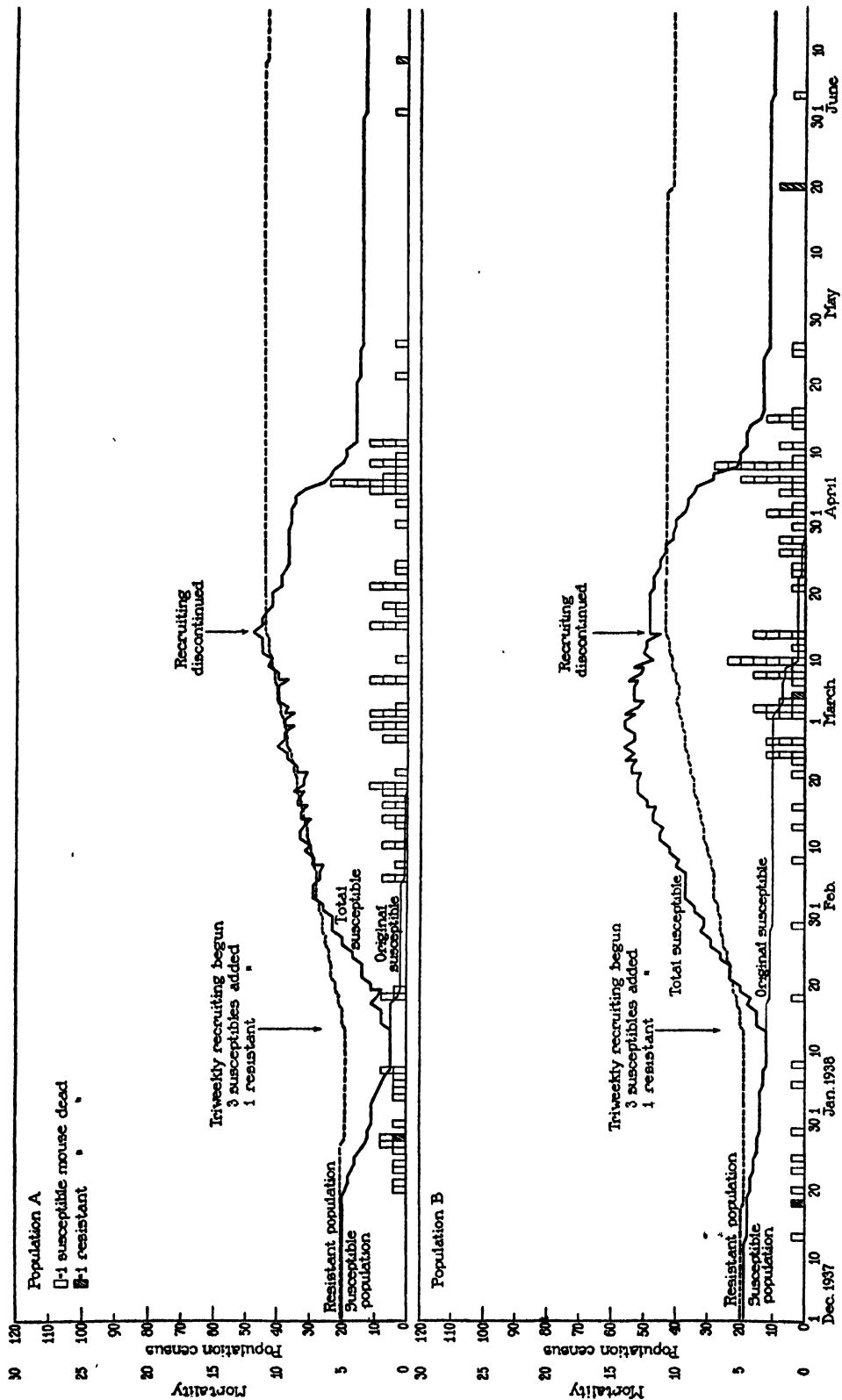
Experiment 3.—Each of four populations, A, B, C, and D, was made up by combining in a single pen twenty susceptibles with twenty resistants, all identified. Population E was set up with thirty resistants plus ten susceptibles and population F with ten resistants plus thirty susceptibles, all properly marked. On Dec. 1, 1937, there were added to each population ten white-face mice, each of which had received the standard dose of mouse typhoid bacilli the previous day. The same routine was then carried out as described in Experiments 1 and 2 and observations continued for 44 days.

The results are shown in Text-fig. 3 and Table I. 80 to 100 per cent of the white-face carriers introduced into the six pens died of mouse typhoid (Table I). Within a few days thereafter, the susceptible contacts commenced dying of typhoid. By the 44th day the epidemics had subsided and mortality had practically ceased. In populations A, B, C, and D mortalities among the susceptible contacts totalled 75, 37, 35, and 85 per cent respectively. In population E the susceptibles, although comprising only 33 per cent of the population, showed a 70 per cent mortality, and in F the susceptibles, 66 per cent of the population, showed a 57 per cent mortality. Of the total 119 susceptible contacts in the six pens, 70 (59 per cent) succumbed.

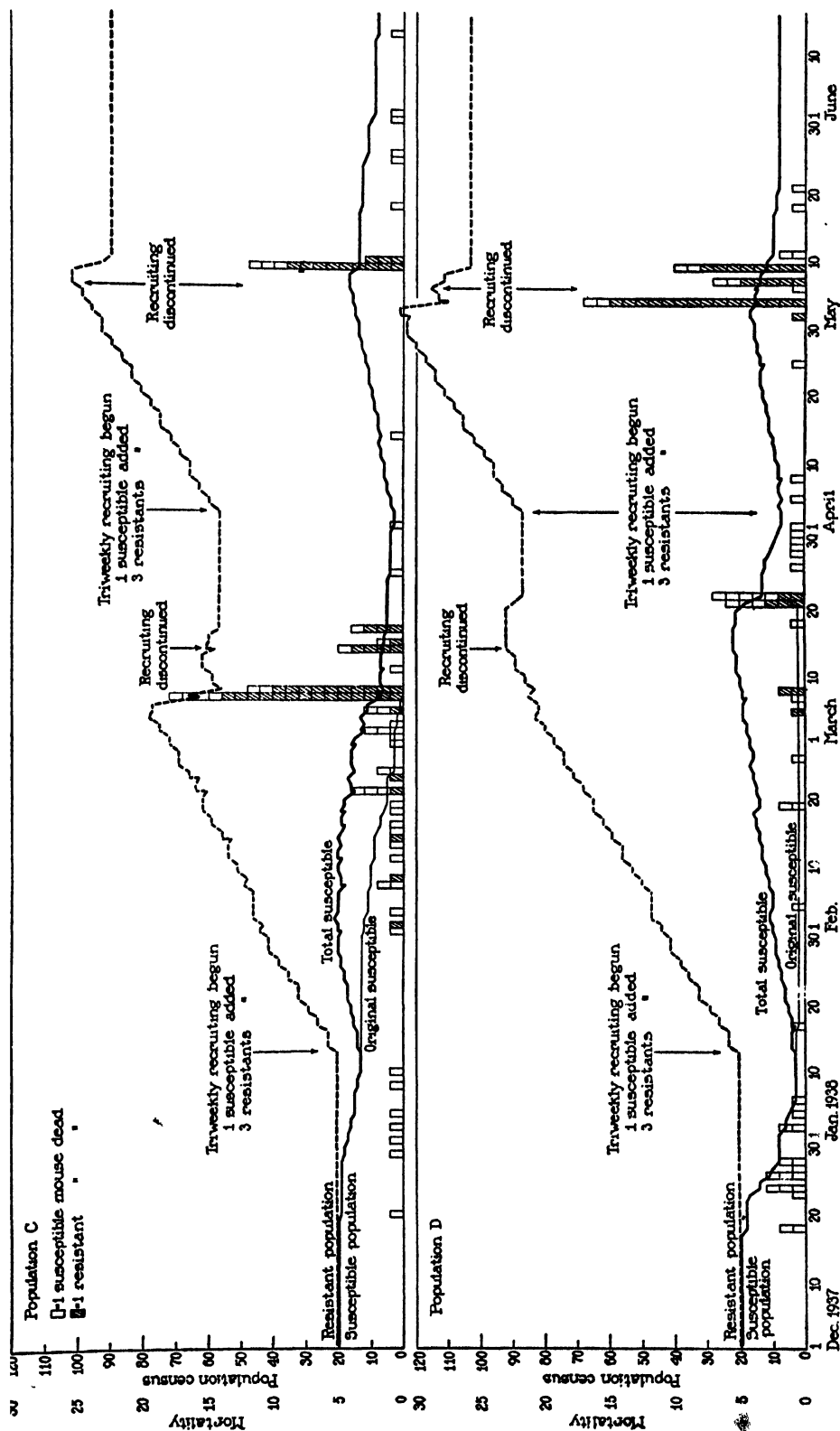
In contrast, only five of 120 resistant contacts succumbed and of these three only died of mouse typhoid (2.5 per cent). This difference shows clearly that in populations with various proportions of susceptibles and resistants, mortality is confined almost exclusively to those contacts known at the outset to be inherently susceptible.

The further fate of these populations is described in Experiments 4 and 5. Population E, however, was observed for an additional 30 days, during which time one more susceptible but no resistant contacts died of mouse typhoid. This population was then discarded.

The next experiment was planned to test the effect of immigration upon

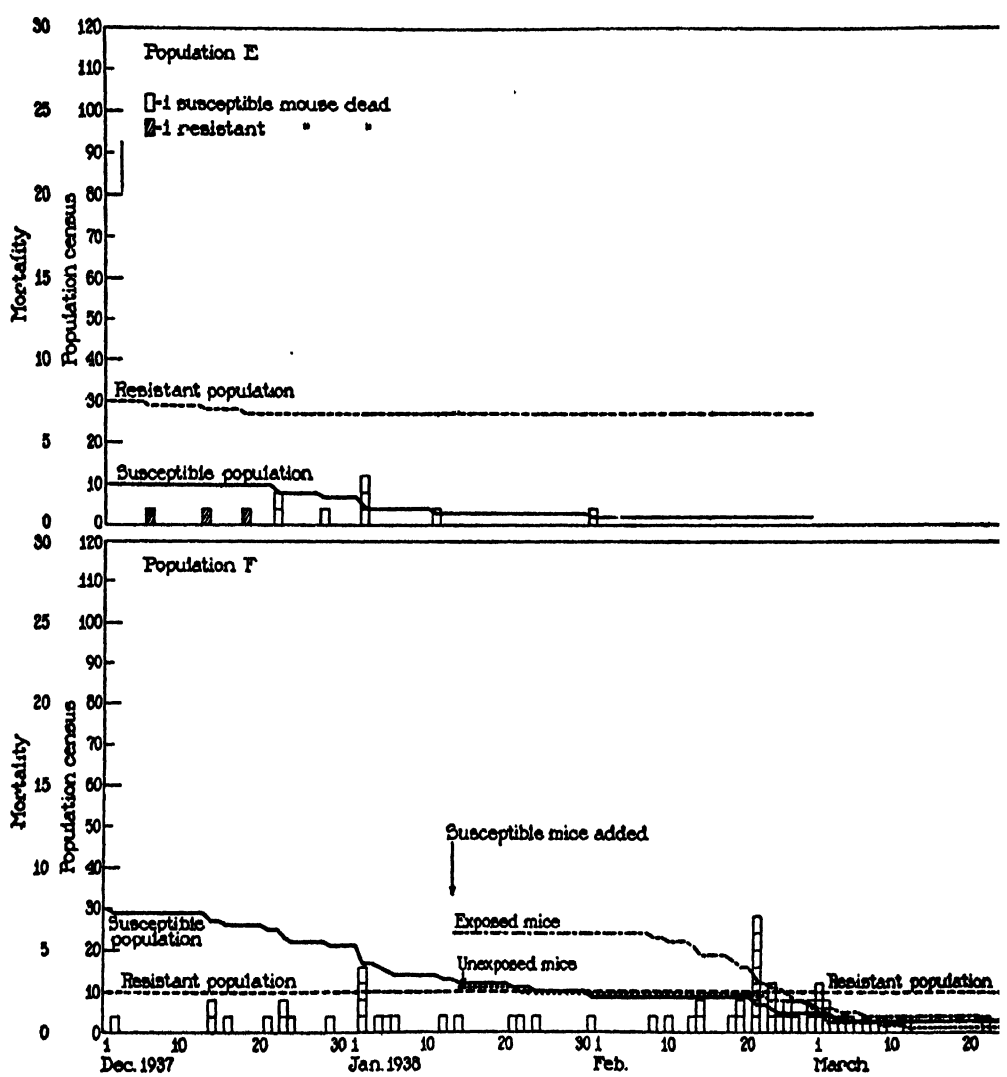


TEXT-FIG. 3—Continued on Next Page



TEXT-FIG. 3—Continued on Next Page

TEXT-FIG. 3. Fate of susceptible and resistant mice exposed to *B. enteritidis* mouse typhoid (Experiments 3 and 4).



TEXT-FIG. 3—Concluded

selective mortality among both the original constituents and the recent recruits.

Experiment 4.—To populations A, B, C, and D recruits were added, commencing on Jan. 13, 1938, 44 days after the populations were first infected. A and B received batches of three susceptibles plus one resistant three times per week, and C and D three resistants plus one susceptible at the same intervals. Recruiting was discontinued on Mar. 14, 2 months later. After another 21 days, on Apr. 4, recruiting was resumed in populations C and D and again discontinued after 31 days on May 6. Observations on all four populations were continued until June 16, a total period of 5 months after recruiting had been commenced. Autopsies and bacteriological identification procedures were carried out on more than 95 per cent of the mice found dead.

TABLE I

Fate of Susceptible and Resistant Mice Exposed to Bacillus enteritidis Mouse Typhoid

Population	Number susceptible		Number resistant		Fate of white-face carriers						Fate of susceptible contacts						Fate of resistant contacts					
					Original number	Final number	Number dead	Number autopsied	Per cent positive	Estimated per cent of total number dead of mouse typhoid	Original number	Final number	Number dead	Number autopsied	Per cent positive	Estimated per cent of total number dead of mouse typhoid	Original number	Final number	Number dead	Number autopsied	Per cent positive	Estimated per cent of total number dead of mouse typhoid
A	20	20	10	2	8	7	100	80	20	5	15	13	100	75	20	19	1	1	100	5		
B	19	20	10	1	9	9	100	90	19	12	7	7	100	37	20	19	1	1	100	5		
C	19	20	10	2	8	7	100	80	20	13	7	6	100	35	20	20	0	0	—	0		
D	19	20	10	1	9	8	100	90	20	3	17	16	100	85	20	20	0	0	—	0		
E	10	30	10	0	10	9	100	100	10	3	7	7	100	70	30	27	3	3	33.3	3.3		
F	30	10	10	2	8	7	100	80	30	13	17	17	94	57	10	10	0	0	—	0		

The results of this experiment are shown in Text-fig. 3 and in Table II. Events in populations A and B differed from those in C and D and hence will be described separately. In A and B, in which triweekly recruiting of three susceptibles plus one resistant was practiced, the census following commencement of recruiting increased relatively steadily until a maximum of 92 and 93 was reached. At this point an equilibrium seemed to be established between number of recruits and amount of mortality. Following the cessation of recruiting, however, mortality still continued at a high rate for about 1 month, reducing the census by more than 50 per cent. Following this few deaths occurred for 2 months.

Throughout the 5 months period, mortality was limited almost exclusively to the innately susceptible mice and these all died of mouse typhoid (Table II). The remaining original susceptibles succumbed within $2\frac{1}{2}$ months, thus failing to show any evidence that they had been im-

munized through exposure. 83 per cent of recruits in A and 87 per cent in B succumbed within 5 months, probably all of mouse typhoid (Table II). In contrast, practically all resistants, both original constituents and later recruits, remained well. One died in A and three in B but not of mouse typhoid.

To populations C and D recruits had been added in the proportion of three resistants to one susceptible. Following this procedure, census levels rose higher than in A and B, to 101 and 114 respectively. At this point the mice were crowded to the utmost and appeared hot, moist, and extremely irritable. Recruiting was discontinued as in A and B on Mar. 14, but within 2 days thirty mice died in C and thirteen in D, apparently from smothering. 2 weeks later, when the survivors seemed adapted to the

TABLE II

Mortality in Mouse Populations Infected with Mouse Typhoid and Recruited with Inherently Susceptible and Resistant Mice

Population	Original susceptibles		Susceptible recruits						Total resistants—Originals plus recruits					
	Number at commencement of recruiting	Number 2½ mos. after commencement of recruiting	Total added	Number remaining	Number dead	Number autopsied	Per cent. positive for mouse typhoid	Estimated per cent of total recruits dead of mouse typhoid	Total added	Number remaining	Number dead	Number autopsied	Per cent. positive for mouse typhoid	Estimated per cent of total recruits dead of mouse typhoid
A	5	0	75	13	62	50	100	83	44	43	1	0	—	0-2.2
B	12	0	75	10	65	60	100	87	44	41	3	3	0	0
C	13	0	40	8	32	24	100	80	140	90	50	26	54	19.2
D	3	0	40	8	32	26	89	71	140	103	37	25	36	3.7

crowded conditions, recruiting was commenced again,—three resistants to one susceptible, as previously. On May 4, with the C population at a maximum of 119 and D at 142, recruiting was again discontinued but not soon enough to prevent the death of fifteen in 2 days in C and thirty-five in 6 days in D. With the populations again reduced to numbers which could survive in the crowded environment and with no further additions to the populations, mortalities practically ceased.

During this 5 month period, mortality in C and D was limited chiefly but not entirely to the susceptible line. Nearly all of these latter died of mouse typhoid (Table II). The remaining original susceptibles were dead within 2½ months after commencement of recruiting, demonstrating again, as in A and B, no tendency to become immunized through exposure to small

doses. Of forty susceptible recruits in population C and forty in D, 80 and 71 per cent respectively succumbed to mouse typhoid. These percentages approximate the 83 and 87 per cent mortalities among each of 75 susceptible recruits in A and B. In C and D, at the close of the 5 month period of recruiting, eight susceptibles remained in C and eight in D.

The resistant line of mice, both originals and recruits, remained well for the most part except during the periods of extreme overcrowding. When the census exceeded 100, however, resistants occasionally succumbed and during the smothering episodes, two in C and one in D, approximately six resistants died to one susceptible. This proportion of 6 to 1 approximates roughly the proportion of resistants to susceptibles actually in these populations, thus indicating that the crowding and smothering hazard was entirely non-specific in its effect, fatal to resistants and susceptibles alike according to their proportionate numbers. At autopsy, on these occasions, 46 per cent of the resistants dying in C and 64 per cent in D showed no evidence of mouse typhoid. Of the remaining positive cases, all but a few showed organisms in the spleen without gross lesions in any organ. Doubtless most of these latter were merely healthy carriers, as observed in Experiment 1. In all, 19.2 per cent of the total 140 resistants in C and 3.7 per cent in D were estimated to have succumbed to mouse typhoid, leaving a surviving population of 90 resistants in C and 103 in D. The virulence of *B. enteritidis* obtained from the spleens of resistants was tested on several occasions according to methods described elsewhere (3). These cultures showed similar mortalities and similar capabilities of spreading and initiating epidemics to cultures obtained from spleens of susceptibles.

Experiment 5.—A final test was made of the ability of susceptible survivors to withstand a subsequent epidemic due to further recruiting of susceptibles. Commencing June 15, and at intervals of 3 to 5 days thereafter, batches of susceptibles were added to A, B, C, and D, until a total of fifty recruits per population was reached.

Epidemics broke out among the susceptible recruits within a few weeks, proving fatal to about 80 per cent. Within 1 month the previous susceptible survivors in C and D were reduced from eight and eight (Table II) to two and one. Similarly, the thirteen and ten in A and B were reduced in 3 months to four and eight. Meanwhile, only three resistants in A, four in B, none in C, and two in D succumbed and these showed no mouse typhoid. The experiment was terminated Oct. 1, 1938.

Taken together, Experiments 3, 4, and 5 show plainly that when populations comprised of known susceptible and resistant mice are infected with mouse typhoid through the introduction of carriers, mortality is confined

almost exclusively to the susceptible constituents, regardless of whether they be present in relatively small or large proportions. Moreover, nearly all of them succumb promptly with no tendency to become immunized and survive. The known resistant constituents, on the contrary, remain well and comprise almost exclusively the surviving population. Again, when populations of survivors, most of them resistants among which no deaths are occurring, are recruited by batches of susceptibles plus resistants in different proportions, mouse typhoid spreads to both classes of immigrants but mortality from the disease is again limited to the susceptibles, whether present in few or relatively large numbers. These latter nearly all succumb within a relatively short time, leaving as survivors only those known to have been resistant at the outset.

In view of the complete failure of susceptible constituents of an infected population to become immunized through prolonged exposure, the effect of repeated short exposures was tested.

Experiment 6.—Population F, at the close of Experiment 3, consisted of thirteen susceptible plus ten resistant survivors. To these mice, some of which were presumably infected and discharging mouse typhoid bacilli in their feces, twenty-five additional susceptible mice were exposed in the following manner. Each was taken from its individual cage and in batches of four was placed in the box with population F for 2 minutes. This procedure was continued mostly daily twenty-nine times. Stool cultures after the sixth exposure contained no typhoid bacilli. One mouse died following the twentieth exposure and was proved to harbor mouse typhoid. This indicated that the immigrants actually had been exposed to the infectious agent.

On Jan. 14 the twenty-four exposed mice plus eleven similar unexposed susceptibles were added permanently to population F to determine whether the exposed mice would prove relatively immune. The contrary proved to be the case. Within 4 weeks an epidemic started among the immigrants which proved fatal in 3 weeks to twenty-one of twenty-four exposed susceptibles (87 per cent) and ten of eleven unexposed susceptibles (91 per cent). Meanwhile, eleven of the thirteen original susceptible survivors had succumbed (85 per cent). In contrast, all of the original ten resistants remained well until the population was discarded 6 weeks later, on Apr. 25.

This experiment shows that repeated short exposure of susceptibles to an infected population, sufficient to bring about a fatal typhoid infection in one individual, nevertheless failed to immunize the remaining to the slightest degree when exposed to an infected yet surviving population in which no deaths were occurring.

A final test was made of the immunizing effect of repeated sublethal doses of mouse typhoid bacilli given *per os* to susceptible mice.

Experiment 7.—Twenty susceptible mice were each given by stomach tube 250 mouse typhoid bacilli in 0.5 cc. of broth daily for 3 days. Subsequently 2,500 organisms were

given daily for 3 days. Stool cultures made each day for 10 days were positive in nine of the twenty mice. 3 weeks later, all were given 500,000 mouse typhoid bacilli by stomach tube. Twenty unexposed controls received the same test dose.

Within 3 weeks, both groups had succumbed to the test dose which was not greater than 10 M.L.D.

The experiment was repeated with thirty mice in each group. Those exposed received double the above number of organisms and four of the thirty succumbed. Twenty-one of the twenty-six survivors showed positive stools. 3 weeks later, following the test dose, none of the exposed nor controls survived.

These experiments failed to show that sublethal doses of mouse typhoid bacilli administered to mice *via* the normal portal of entry immunized them to a subsequent test infection by mouth.

The same results were obtained in mice given repeated nasal instillations of a sublethal dose of St. Louis encephalitis virus followed 4 weeks later by a test nasal instillation of 10 M.L.D.

DISCUSSION

By using mice whose individual susceptibilities, both innate and environmental, are known and controlled, direct experimental data have been obtained for the first time on the following epidemiological problems.

Fluctuations in infectivity and virulence in infected mouse populations as investigated thus far prove to be manifestations of differences in host resistance rather than in parasite potentialities. Thus, in the foregoing experiments, plus others reported earlier (1, 3), a single strain of mouse typhoid bacilli spread equally to resistant and to susceptible mice but showed at the same time a high killing potency in susceptibles and a low killing potency in resistants. Altering resistance through diet (3) alters killing potency but not infectivity. On the other hand, when host resistance factors are kept constant and uniform through the use of mice of known susceptibility, infectivity and virulence likewise remain constant.

Mortality from mouse typhoid in exposed or infected populations is conditioned by the number of highly susceptible constituents. If the number is few, deaths are sporadic; if great, epidemic; if susceptibles are depleted, mortality subsides. Previously it was known that fresh mice added to an infected population initiate or maintain mortality at epidemic levels, but their exact rôle has remained uncertain. Topley and Greenwood who considered the fresh mice to be alike in their individual susceptibilities furnished no experimental data as to their function. We found that the fresh mice differed in their initial susceptibilities (1) but were in doubt as to whether these individual differences or the average level of susceptibility was the more important. It now appears that the presence of the in-

dividual with high susceptibility initiates the outbreak, no matter what the general level of susceptibility may be. If these individuals are few in number, mortality remains sporadic; if numerous, it becomes and remains epidemic. Finally, if and when the susceptibles are depleted, mortality subsides. Thus the danger to an already infected population resides not alone in a general fall of the resistance level but in an immigration of highly susceptible individuals.

A proportion of survivors are infected and remain the reservoir from which the infectious agent spreads to incoming susceptibles.

Clearly from these experiments, survivors are the initially resistant constituents and there is little or no tendency for susceptibles to become immunized through exposure to chance small doses.

At this point it may be objected that these populations of extremely resistant plus susceptible individuals have no counterpart in nature and hence that their behavior is not analogous to that of populations comprised mainly of individuals of intermediate grades of susceptibility. In reply we argue that the extreme individuals are in reality closely related both to one another and to the moderately susceptible individuals (2). In some cases the relation is that of siblings—in all events, the differences are those which may occur normally among individuals of the same family. The behavior of infected populations comprised solely of individuals with extreme differences in susceptibility has not differed qualitatively in so far as we have observed from populations comprised of individuals with all grades of susceptibility (3). We conclude, therefore, that the amount of resistance of each individual at the outset determines whether it will survive an epidemic of mouse typhoid. If it survives, there is a possibility, not yet demonstrated experimentally, that it may develop, through infection, what might be termed a luxury immunity.

Reinoculation of survivors, as ordinarily practiced, is not a test of active immunity. Armstrong, for example, reports that since mice surviving nasal instillation of St. Louis virus are relatively resistant to a second instillation, they have been immunized (5). Such a conclusion is unwarranted without knowledge of whether some animals were resistant at the outset or whether all were susceptible. We have been unable to immunize known susceptible mice by instilling nasally sublethal doses of St. Louis encephalitis virus. In our opinion a test for active immunity must be made on batches of animals known to be at least 90 per cent susceptible to the test agent given by a normal portal of entry.

Continued resistance of survivors does not depend necessarily upon the

presence of bacilli in the tissues. If initial resistance is high, bacilli persist indefinitely in the spleen without harm and without altering the already adequate level of resistance.

In the foregoing experiments we are mindful that the resistance actually displayed by the individual was a summation of inherent components which were altered experimentally, plus environmental ones associated with age, regimen, and diet which were kept constant. Moreover, we know by experiments under similar herd conditions that diet exerts equally important effects in epidemics under conditions in which inherited components are kept constant (3). The main point, after all, is that inherited and general environmental components of resistance are of fundamental importance, whereas immunological components associated with infection are of negligible importance in mouse typhoid infection.

This finding that inherited resistance factors exercise a fundamental control, whereas specific immunity factors play a minor rôle in mouse typhoid, is at present of limited application. It would hold, we believe, among both individuals and populations under the following circumstances: (a) infections gaining access and spreading for the first time, (b) infections associated with high mortality rates, and (c) infections with low morbidity rates. In infections with high morbidity and low mortality rates, however, we would look for specific immunity factors to be important in preventing recurrence. Pending experiments on this latter type of infection, one can state merely that the theory of immunity through sub-clinical infection has been overextended and that misleading conclusions have been drawn from the classical experiment of reinoculating survivors.

CONCLUSIONS

1. Under conditions in which mouse typhoid is allowed to spread naturally among herds of mice comprised of different proportions of individuals of innately high or low susceptibility: (a) 85 to 95 per cent of the innately susceptible succumb to mouse typhoid in contrast to less than 5 per cent of the innately resistant, regardless of whether either constitutes 25, 50, or 75 per cent of the population respectively. (b) The surviving population is therefore comprised largely of individuals known at the outset to be innately resistant. These resistants are, nevertheless, apt to have become infected and to harbor mouse typhoid bacilli in their spleens and feces.

2. Under conditions in which recruits are added to surviving populations comprised chiefly of innately resistants among which mortalities have practically ceased: (a) Mouse typhoid infection spreads to both innately

resistant and susceptible recruits. (b) Mortality from mouse typhoid is limited almost exclusively to the innately susceptible recruits and is "sporadic" or "epidemic" in character according to the numbers and proportion of susceptibles added. (c) Innately resistant recruits remain well unless subjected to some non-specific hazard, such as heat or overcrowding, in which case both they and the susceptibles succumb in proportions similar to their relative numbers in the population.

3. It was plain that survivors are almost exclusively the individuals known at the outset to have been innately resistant.

4. There was no tendency for known susceptibles to become immunized through herd exposure at epidemic times, at postepidemic times in which the dosage of mouse typhoid bacilli was relatively small, nor at repeated short intervals. Finally, susceptibles given repeated, known, sublethal doses of mouse typhoid bacilli or St. Louis encephalitis virus by a natural route failed to develop immunity against a subsequent test dose.

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A GENERAL PERMEABILITY-INCREASING EFFECT OF A FACTOR FROM MAMMALIAN TESTICLE ON BLOOD CAPILLARIES*

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It is known that the spreading factor has a pronounced effect in increasing the permeability of the skin connective tissue, as is shown by the enhancement of infections³ and the spreading of colored solutions or suspensions when the infectious agent or the colored matter is injected intradermally along with spreading factor from various sources, such as extracts of mammalian testes,^{11,12} invasive bacteria,⁷ snake⁴ and other animal⁹ venoms, leech extracts,¹ etc. Adequate injections of colored solutions or suspensions mixed with spreading factor into muscles, tendons, the walls of stomach and intestines, etc. result, too, in a pronounced spreading.^{5,8} Therefore, we are dealing with a general phenomenon affecting the connective tissues of many organs. On the other hand, several facts have been disclosed showing that the permeability of the vascular system can be locally or generally increased by means of preparations rich in spreading factor. Such facts can be grouped as follows:

(a) *Local Increase of the Capillary Permeability from Without*

Viruses³ and dyes⁴ injected intravenously in the rabbit are promptly localized in the areas of skin injected with extracts of testicle and snake venom respectively.

(b) *Local Increase of Capillary Permeability from Within*

A practically immediate "preparation" of the tissue for the occurrence of the Shwartzman phenomenon is obtained when the active bacterial filtrate mixed with testicle extract is injected into the vascular system of the rabbit ear in which circulation has been temporarily stopped.¹⁴ Filtrate alone, or mixed with a variety of other substances known to be active on blood vessels, has no such effect.

* The present investigation was started in The Rockefeller Institute, New York. Aided in part by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

(c) General Increase of Capillary Permeability from Within

Preparation of the kidney for the Shwartzman phenomenon is obtained when the active bacterial filtrate mixed with testicle extract is injected intravenously. In such case the responsible testicle factor has presumably exerted an action on all blood capillaries, but only those of the kidney, due to inherent conditions of its own vascular system, have shown the typical hemorrhagic reaction on reinjection of the filtrate.¹⁵

When testicle extract, an extract of invasive staphylococcus, or rattlesnake venom is injected intravenously in the rabbit and then vaccine virus or India ink is injected intradermally a more pronounced lesion¹² or a larger spread^{4, 7} will result. If the rabbit has been prepared for a period of three weeks by several intravenous injections of testicle extract and as late as two weeks after the last dose the skin is injected with vaccine virus a markedly spreading lesion ensues.¹³ This was accidentally found by Parker and Rivers and was interpreted by them as due to the action of the spreading factor. This finding is interesting, since it shows that a state of generally increased capillary permeability, presumably induced by the spreading factor, can last for a considerable period.

When rabbits bearing in their skin large lesions produced by invasive strains of staphylococcus—very active producers of spreading factor—are injected, into distant areas of the same organ, with suspensions of India ink a spreading of the injected material takes place. The same phenomenon is observed in rabbits bearing pronounced staphylococcal lesions in the testes. In the latter case the spreading factor is clearly detected in the blood.⁷

Repeated observations have shown that there is always a parallel between the degree of increase of connective tissue permeability (skin spreading) and the degree of increase of vascular permeability (skin localization). This is especially true in the case of the highly purified and very active preparations of testicle extract. Although a final statement on the matter must await the chemical isolation of the active pure fraction, one may temporarily assume that the effects on the connective tissue and on the blood capillaries are manifestations of the same substance or of two substances very closely linked.

In the present investigation we have studied the effect on the whole capillary system of active preparations from testicle by injecting them, together with dyes, intravenously and judging the effect on the permeability of the blood capillaries by the degree of discoloration shown in the corresponding tissues.

Materials and Methods

Testicle extract: Purified preparations of bull testes, prepared by a method previously described,³ were generally used. The fresh gland, trimmed of its membranes, is passed through a meat-grinder. It is then extracted with an equal

volume of 0.1*N* acetic acid and the mixture is left standing over night in the refrigerator. After filtration through paper, the spreading factor is precipitated by 4 volumes of acetone and the precipitate is washed in a Buchner funnel with more acetone until free of water. After drying in air the yield of dry powder amounts to from 1.15 to 1.50 gm. per 100 gm. of fresh tissue. The powder is only partly soluble in H₂O, but practically all the spreading factor goes into solution on extraction with this solvent. The powder, kept in the refrigerator, is extracted with saline solution, generally at the ratio of 1:10. When it is prepared for use, the mixture is centrifuged and the supernatant fluid used. When tried on the rabbit skin such preparations still caused spreading of the India ink at dilutions of 1:100,000. In some experiments rat testes from recently killed animals were ground with sand in 3 or more volumes of saline. The resultant pulp was centrifuged and the supernatant used. Whatever extract was used, prolonged centrifugation and elimination of any suspended particles by filtration was found necessary to obtain preparations devoid of toxicity on intravenous injection.

Dyes: Three azo dyes have been employed: *T.1824*,* acid, rather heavy and poorly diffusible, manufactured by Eastman Kodak Co.; *Congo red*, also acid, and poorly diffusible; and *Bismarck brown*, basic, very diffusible. The three dyes were used diluted in saline at the ratio of 1:100.

Animals: Mice from the Swiss and The Rockefeller Institute strains and guinea-pigs have been used.

Method: The solution of dye was injected intravenously in mice or guinea-pigs, either mixed with testicle extract or at different intervals after injection of the latter. The animals were killed by decapitation or by chloroform at varying periods after injection. They were autopsied immediately and the degree of tissue discoloration was studied while the organ was immersed in water. The strong blue color of *T.1824* allowed accurate comparative observations. When Congo red was used tissue discoloration was studied before and after immersion in a normal solution of HCl. Under the influence of the acid the original red color turns blue and the detection of the dye in the tissue is thus much facilitated.

EXPERIMENTAL

Tests on the Permeability of Blood Capillaries of Tissues in General

In the first part of the investigation determinations were made of the effect of testicle extract on capillary permeability during the first 24 hours after intravenous injection of the extract mixed with dye.

Experiment. In different tests 30 mice, each weighing from 15 to 20 gm., were used. Half of them were injected in the vein with 0.25 cc. of 1:10 purified extract

* A similar preparation also made by Eastman Kodak Co. is now on the market under the name of Evans blue.

of bull testicle in saline solution mixed with an equal volume of 1 per cent T.1824 solution. As controls, the other half of the mice were similarly injected, using saline solution instead of testicle extract. At intervals ranging from 30 minutes to 24 hours after the injection, pairs of mice from test and control series were killed. Autopsies were done immediately and the degree of discoloration of corresponding tissues of both test and control mice was compared under water.

Results in general can be summarized as follows: The skin of mice injected either with testicle extract or saline mixtures turned slightly blue immediately after injection. However, soon after, the discoloration of the mice injected with testicle extract increased to a deep cobalt blue, while the control mice showed only a pale blue tinge. Such differences were most clearly shown in the ears and pads. Examination of tissues after death showed that as early as 15 minutes after injection the internal surface of the skin, the lungs, muscles, and liver of the mice injected with testicle extract were much more discolored than were the corresponding tissues of the control mice. In other tissues the differences were less constant and less marked. Thus, kidneys showed clear differences in 5 cases, lymph nodes in 6, intestines in 3 cases. In other tissues only occasional differences were found. The central nervous system was never stained in either kind of injected mice.

The above experiments were complemented by some tests in which Congo Red, instead of T.1824, was used as indicator.

Experiment. Four mice were injected intravenously with 0.25 cc. of 1:10 purified extract of bull testicle mixed with an equal volume of 1:100 solution of Congo red, and the other mice were similarly injected, substituting saline for the testicle extract. The animals were killed either 15 minutes or 18 hours after the injection and the degree of discoloration of the tissue was noted before and after treatment with acid.

The results were the same, in general, as when T.1824 was used and in some cases even more clean-cut, since tissues of the control mice showed in such instances no discoloration whatever, whereas a pronounced discoloration was seen in the corresponding tissues of the test animals.

These tests were then repeated with freshly prepared extracts of rat muscle, brain, and spleen. Such tissues are known to have only moderate amounts of spreading factor. Similar extracts from rat testes were also prepared.

Experiment. Muscle, brain, spleen, and testes from rats recently killed were each extracted with 10 or 20 volumes of saline solution. In different tests, approximately 25 mice were injected in the vein with mixtures, in different proportions, of the tissue extracts plus 1 per cent solution of T.1824, while as many controls were injected with the dye plus corresponding amounts of salt solution. The

amounts of tissue extract varied from 0.1 to 0.4 cc. and the amount of dye from 0.1 to 0.3 cc. The injected mice were killed after 2 to 4 hours following injection. The results can be summarized as follows: Shortly after inoculation the skin of most mice injected with testicle extract mixtures became bluer than did the skin of the corresponding controls, and when the mice were sacrificed the tissue of those injected with testicle extract mixtures showed more pronounced discoloration than did the corresponding tissues of the control mice. The differences were analogous, although not so marked, to those described in the foregoing experiments in which purified testicle extract was used.* On the contrary, mice injected with dye mixed with extract of muscle, brain, or spleen did not show any increased discoloration when compared with corresponding tissues of the control animals.

Such tests justify the conclusion that bull or rat testicle extract injected with solutions of T.1824 or Congo red enhances the speed of passage of the dye from the circulation to the tissues, as judged by their more pronounced discoloration as compared with that shown by tissues of mice injected with dye alone. This effect is generally apparent within from a few minutes to 24 hours after injection.

The next step was to determine the duration of the state of increased dye migration from blood to tissues brought about by testicle extract. The general procedure in 4 experiments consisted in the preparation of one group of mice by intravenous injections of testicle extract and another group by similar injections of saline. Beginning 24 hours after such injections and for several successive days a mouse from each group was injected in the vein with dye solution. The 2 animals were sacrificed 24 hours later and the degree of discoloration of their tissues was compared. The results, and the details of the technic, are summarized as follows:

Experiment. Each of 6 mice was prepared with 0.3 cc. of 1:3 extract of freshly obtained rat testicle. There were 6 controls. These mice were injected daily for 5 successive days with 0.1 cc. of 1 per cent T.1824. In every case the tissues

* Evidence derived from other tests similar to those above described has shown that the effect induced by fresh extracts of rat testes may be less prolonged and constant than is the effect induced by purified preparations of bull testes. Cases may be found where clear enhancement of the tissue discoloration is seen when the mouse is killed shortly after the intravenous injection of dye plus freshly prepared rat testicle extract, but no such enhancement is seen when the mouse is killed 24 hours later. Differences in strength or amount of the active factor probably account for such variations.

of the mice prepared with testicle extract looked much bluer than did those of the corresponding controls. Such differences were very conspicuous in the liver, kidneys, intestines, and lymph nodes; less so, although quite marked, in skin and muscles.

Experiment. Nine mice were prepared with 0.25 cc. of 1:10 purified extract of bull testicle. These, with 9 control mice, were injected on the following days with 0.25 cc. of 1 per cent T.1824. External examination of the mice shortly after injection showed that up to the 4th day the skin of the mice prepared with testicle extract turned much bluer than did the skin of the control mice. However, when the mice were sacrificed 24 hours later no differences in tissue discoloration of the 2 groups were found, except in the skin of ears and pads which were more discolored in the mice prepared with testicle extract. From the 4th to the 7th day no more immediate differences were found after the injection of dye, and autopsies 24 hours later showed that the tissues of the control mice were actually more discolored than were corresponding tissues of mice injected with testicle extract. From the 7th to the 10th day no differences of any kind were observed between mice of both groups.

Experiment. Eight mice, prepared with 0.25 cc. of 1:10 purified extract of bull testicle, and as many controls, were injected on the following days with 0.25 cc. of 1:100 T.1824 solution. During the first 4 days the skin of the mice prepared with testicle extract turned bluer than did that of the controls shortly after the intravenous injection of dye. After this time no more immediate external differences were noticed. The differences in the discoloration of tissues when the mice were sacrificed 24 hours later are expressed in Table I.

The results as presented in the table and the other two experiments described above show that following an intravenous injection of bull or rat testicle extract into mice, a dye injected by the same route within the 4 or 5 succeeding days migrates from the blood into the tissues much faster than when the dye alone is injected. After this time the results obtained suggest that for a period of 2 or 3 days the dye is present in less amount in tissues of those mice receiving the testicle extract than in those of the controls, but in order to reach a final conclusion on this complex point more work is required. After the 7th day no difference could be noticed in the amount of dye present in the tissues of mice of both groups.

Tests on the Permeability of the Blood Capillaries of the Central Nervous System

In the experiments described above the central nervous system of the mice injected, with or without testicle extract, was practically

TABLE I
Comparative Discoloration of Tissues in Mice Previously Injected with Either Testicle Extract or Saline Solution and Re-injected with T.1824 Solution

Time between injection of testicle extract or saline solution and injection of dye	Skin		Muscles		Lungs		Liver		Testes		Intestines		Lymph Nodes	
	T.E.	Sal.	T.E.	Sal.	T.E.	Sal.	T.E.	Sal.	T.E.	Sal.	T.E.	Sal.	T.E.	Sal.
1 day....	+++±	++	+++++	++	+++	++	+++++	+++	+++	±	+++	±	+++	++
3 days....	+++++	+++	+++++	++	+++	±	+++++	+++	+++	+	+++	+	+++	++
4 days....	+++	++	++	+++±	+	++	+++	+++±	+++	+	+++	+	+++	++
6 days....	+	+++	+	+++	+	+++	+++	+++	+	+	+	+	+++	++
7 days....	±	+++	+	++	±	+++	+++	+	+	+	±	+++	+++	++
7 days....	±	++	+	++	±	++	+++	++	++	+	±	++	++	++
10 days....	++	++	+	+	+	+	+++	+++	++	+	+++	+++	+++	++

Results recorded 24 hours after the intravenous injection of 0.25 cc. of a 1% solution of T.1824.

never stained by the dyes used—Congo red and T.1824, two acid dyes. It has been shown by Friedemann¹⁰ that all basic anilin dyes, with the exception of safranine, stain the brain when injected in small amounts, while acid dyes do not stain this tissue at all or only when large amounts are injected. Trypan blue, a dye similar to T.1824 and Congo red, falls into the latter group.

It was desirable to know whether testicular extract also enhanced the passage into the central nervous system of basic dyes having under normal conditions an affinity for it.

Experiment. In preliminary tests mice were injected intravenously with 0.4 cc. of 1 per cent solution of Bismarck brown and killed by chloroform at different periods ranging from a few seconds to 24 hours. The brain appeared most stained when the mouse was killed immediately after injection and was colorless when killed after 24 hours.

Nine mice were then injected in the vein with a mixture of 1 per cent Bismarck brown and purified extract of bull testicle. The amount of extract used varied from 0.25 cc. to 0.5 cc., the amount of dye from 0.25 cc. to 1.5 cc. Another set of mice were similarly injected with similar mixtures of saline solution and dye. The mice from each series were killed with chloroform in from 5 to 15 minutes after injection. In 5 cases the brain tissue of the mice injected with testicle extract was more discolored than was that of the control animals, while in the other 4 cases no differences were noticed. The differences in discoloration in the 5 positive cases were very pronounced in 2 instances, slightly so in the other 3.

These tests show that testicle extract may enhance the passage of Bismarck brown from the blood into the central nervous system, but apparently the phenomenon is not constant in the mouse. It is difficult to explain such a lack of regularity, although the short duration of the staining of the tissue by such a diffusible dye and the small size of the injected animal may be important contributing factors.

The experiments were then repeated on guinea-pigs, the species used by Friedemann in his tests.

Experiment. Four guinea-pigs, each weighing about 300 gm., were injected in the heart with mixtures of 1 per cent Bismarck brown solution and 1:20 purified preparation of testicle extract. The amounts of each ingredient are expressed in Table II. The animals were killed within 4 to 5 minutes after injection and the central nervous system was exposed. The degree of discoloration of the nervous matter is shown in Table II.

It is clear that in every case the addition of testicle extract to the inoculum has pronouncedly enhanced the passage of the injected Bismarck brown from the blood to the central nervous system.

TABLE II

Enhancement of the Passage of Bismarck Brown from the Blood into the Brain by Means of Testicle Extract

(Results 5 minutes after injection)

Guinea-pig number	Mixture injected	Resultant discoloration in brain
1	T.E.—0.25 cc. plus B.B.—0.25 cc.	±
2	Sal.—0.25 cc. plus B.B.—0.25 cc.	—
3	T.E.—0.50 cc. plus B.B.—0.50 cc.	+
4	Sal.—0.50 cc. plus B.B.—0.50 cc.	±
5	T.E.—1 cc. plus B.B.—1 cc.	+±
6	Sal.—1 cc. plus B.B.—1 cc.	±
7	T.E.—2 cc. plus B.B.—2 cc.	++±
8	Sal.—2 cc. plus B.B.—2 cc.	+

T.E. = Testicle extract 1:20

B.B. = Bismarck brown 1%

In this experiment, as in the tests with mice described before, paralleling the differences of staining of the central nervous system,

some of the other tissues, including fat tissue, of the animals injected with the testicle extract mixtures were more discolored than were the corresponding tissues of the control animals.

Tests on Cancer-Bearing Mice

The purpose of these studies was to investigate whether the abnormally high ability to localize circulating dyes demonstrated by us⁶ in cancer tissue could be enhanced still further by means of testicle extract.

Experiment. Twelve mice bearing transplantable sarcoma S/37 or 180 were injected in the vein with variable amounts of solutions of purified testicle extract and T.1824, and a like number were similarly injected substituting saline for the testicle extract. On sacrificing the animals at different intervals after injection no differences in the amount of dye localized in the tumors could be found.

Tests on the Influence of Age

In all the tests carried out in the present investigation special attention was paid to the age of the mice with the view of ascertaining possible differences in the ability of tissues from young or old individuals to localize the circulating dye injected, mixed either with testicle extract or with saline. Moreover, some additional tests were devised in which young and old mice were proportionally injected with dye mixed with saline or testicle extract. No differences were found. *It may be worth noting that old mice are apt to have chronic infections or spontaneous tumors. Such processes bring about a pronounced localization of the injected dye in the affected area and its surroundings.*⁶

DISCUSSION

The experiments reported here show that if a dye normally having a staining effect for a given tissue when introduced in the circulation is injected into the blood stream together with testicle extract the staining effect for that tissue is very much increased. This is judged by the more pronounced discoloration of such tissue as compared with the corresponding control when the animals are sacrificed at different periods after injection. The simplest explanation of these facts is that the permeability of the blood capillaries has been increased

by the testicle extract, thus allowing a more rapid passage of dye from the blood into the tissues. Which is the active factor in the testicle extract responsible for such effect? In the introduction to this paper we have noted the cases in which several preparations very active in increasing the permeability of the connective tissue exert, also, a pronounced effect on the permeability of blood vessels, suggesting that the spreading factor may be responsible for both effects. The same suggestion seems logical in the experiments here reported. Only extracts from testicle have been found active, while extracts from muscle, brain, and spleen having little spreading factor were inactive. Extracts from bull testes purified from much inert matter but keeping practically all the spreading factor are especially effective in increasing the permeability of the blood capillaries.

The factor from testicle extracts which increases vascular permeability does not alter the specificity or selectivity in the localization of dyes in tissues. Acid dyes, like Congo red or T.1824, which under normal circumstances do not localize or localize but very little in the central nervous system, are not altered in their tissue affinities when injected together with testicle extract. On the contrary, the normal staining capacity of Bismarck brown, a basic dye, for the central nervous system is much enhanced by a simultaneous injection of testicle extract. In this respect, too, the active factors behave like the spreading factors acting on skin infections. It has been said¹⁶ that the effect of the spreading factor was like the effect of a magnifying device rather than a promoter of new conditions. The same principle would apply, too, to the factor increasing the permeability of blood capillaries. The importance of such effect on the generalization of infection or intoxication seems clear.

If the factor acting on blood capillaries were conclusively shown to be identical with the spreading factor, then the problem of the permeability of blood capillaries could be studied under the light of existing knowledge of the action of the spreading factor on connective tissue.

SUMMARY

When solutions of acid T.1824 or Congo red are injected intravenously together with testicle extract the speed of passage of the

dye from the blood into the tissues is pronouncedly increased. However, the central nervous system, not stained by these two dyes under normal conditions, remains also impervious to them when injected together with testicle extract.

When solutions of basic Bismarck brown are injected intravenously together with testicle extract the speed of passage of the dye from the blood into the central nervous system is also pronouncedly increased.

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A NOTE ON THE ACTION OF SOME CARCINOGENIC HYDROCARBONS ON AMPHIBIA*

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If a causative virus can be demonstrated in mesenchymatous fowl tumors, either in those originating spontaneously⁴ or induced by chemical carcinogens,³ and if at least two other viruses are the probable causes of two epithelial tumors, one in the frog (*R. pipiens*)² and the other in the Triton (*T. alpestris*),¹ it would obviously be interesting to ascertain whether viruses could be demonstrated in chemically induced tumors in amphibia. Unfortunately, despite many attempts, no such tumors have been induced. However, some points of a minor interest have been brought to light and the description of such findings is the object of the present note.†

Experimental. In 4 successive years 1935-1938, 261 frogs belonging to the species *Rana pipiens*, *Rana clamitans* and *Rana catesbiana* were inoculated with hydrocarbons highly active in inducing malignancy in certain mammals. About 75 more frogs served as controls of different experimental factors. Moreover, 18 newts (*Triturus pyrrhogaster* and *Triturus viridescens*) were similarly inoculated.

The frogs were injected in the thigh muscles with 1:2:5:6 dibenzanthracene, benzpyrene, and methylcholanthrene in amounts ranging from 0.125 mg. to 4 mg. The chemicals were generally dissolved in lard, but they were also used in colloidal suspension in gelatin solution or horse serum.‡ The newts received 2 mg. of dibenzanthracene solution in lard. The frogs were kept in jars containing wet moss (a) in the laboratory; (b) at outside temperature; (c) in the incubator regulated at 39°. They were given beef liver in forced feeding once or twice a week. The experiments were conducted throughout the whole year. Details and some results of the experiments on frogs are given in Table I.

* This study, started at the Rockefeller Institute in New York, was aided in part by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

† Champy and Vasiliu in 1924 also attempted in vain to induce tumors in tritons by prolonged tar painting. Ulcers and cachexia developed but animals never showed tumors.

‡ The horse serum preparations were kindly supplied by Dr. H. B. Andervont.

Post-mortem examination and histological study of the tissues of the dead animals showed that the immediate effect of the chemical was essentially necrotizing, the lesions being characterized by degeneration of the muscle fibers, edema, hemorrhage, fibrin deposition, and scanty cell infiltration. Analogous lesions were sometimes seen

TABLE I
Survival of Frogs after Inoculation with Carcinogenetic Hydrocarbons

Year	Surrounding temp. of inj. frogs	Carcinogen injected	Species of Rana	Total number of frogs	Incidence of death after injection							
					20 days	Months						
						1	2	3	4	5	6	7
1935-36	Laboratory	From 0.125 to 4 mg. D. B. A.	pipiens clamitans catesbiana	140	62	29	28	8	10		2	1
1937-38	Incubator	2 mg. D. B. A.	pipiens	15	10	2	1	2				
1937-38	Outside	2 mg. D. B. A.	pipiens	11	1	3	3	3				
1937-38	Incubator	2 mg. D. B. A.	pipiens	8	8							
1937-38 ..	Outside	2 mg. D. B. A.	pipiens	40	5	11	10	6	4	2	1	1
1937-38	Incubator	2 mg. B. P.	pipiens	11	11							
1937-38	Incubator	2 mg. M. Ch.	pipiens	19	18	1						
1937-38	Incubator	1 mg. M. Ch.	pipiens	17	9	3	1	1	3			

in the uninjected thigh. Some weeks later fibrosis and proliferation of the vascular endothelium were observed co-existing with increased muscle necrosis. Still later, fibrous pockets were found containing crystals of the hydrocarbon suspended in a fluid mass of lard and cell débris. Conspicuous bloody edema of both hind legs was often seen. The animals were very emaciated. At the same time

that local lesions were developing the animals showed generalized lesions characterized by congestion, hemorrhages, and necrosis in spleen, liver, kidney, testes, and lung. These were already detectable a few days after injection. Subcutaneous and peritoneal lymph was increased in amount and was hemorrhagic. Intravascular hemolysis was frequent. Later, one observed lymphocytic depletion in the spleen and disappearance of the spermatogenesis. Pigment-loaded phagocytic cells in lung, liver, and spleen were much increased in number. In frogs injected with lard solution all of these changes, local as well as general, were more pronounced and appeared much earlier (3 to 4 days) in individuals kept in the incubator than in those kept at outside or room temperature. In the latter groups most of the lard was found in the injected thigh many weeks after inoculation, whereas in the former group it disappeared in a few days, thus presumably allowing the hydrocarbon to exert its action. The same was the case with frogs injected with the colloidal suspension of dibenzanthracene.

Of the 3 hydrocarbons used methylcholanthrene and benzpyrene proved to be more active in inducing earlier and more pronounced changes than was dibenzanthracene. Of the 3 frog species used *R. pipiens* and *R. clamitans* were found, in the same respect, more susceptible than was *R. catesbiana*.

It is well known that mortality is high in frogs kept in captivity and, as Table I shows, it was still higher in our injected frogs kept in the incubator. However, the uninjected control frogs kept in the incubator died, too, in a large proportion and when such frogs were injected with lard, horse serum, or cholesterol, or were grafted with other frogs' tissue the mortality rose as high as that in the frogs injected with hydrocarbons. Bacterial or parasitic conditions were in many cases the obvious cause of death. Several of such control animals were carefully studied after death. Most of them showed generalized lesions consisting of congestion, hemorrhage, and necrosis in the kidneys, liver, and possibly (one case) the testicle. Such lesions were similar to those seen in the frogs injected with carcinogens. Only the spleen changes occurring in the latter animals were not encountered in the controls. Therefore, it would be extremely unsafe to

attribute to the action of the chemical all of the generalized lesions observed in the injected frogs kept in the incubator. As for the generalized lesions shown by the injected frogs kept at outside or room temperature, we lack control animals kept long enough under the same conditions to justify final conclusions. However, the impression gained was that the lesions seen in animals under such conditions are more in direct dependence on the hydrocarbon injected than when frogs were kept in the incubator. For instance, a careful investigation carried out in 6 frogs kept in captivity for at least 10 days at outside temperatures failed to disclose even the slightest alteration in their tissues. Concerning the local lesions in the injected thigh it seems quite reasonable to attribute them to the action of the chemical and the same could be said of the lesions in the opposite thigh where, through the lymph, the injected material has easy access.

Of the 18 newts injected with dibenzanthracene 9 died within the first month, 5 within 2 months, and 4 within 3 months. Degenerative changes analogous to those seen in frogs were observed in the injected muscles. No systematic study was made of other tissues.

For the following reason special attention was paid to the incidence of the Lucké adenocarcinoma in the kidney of *R. pipiens* in individuals injected with carcinogens as compared with controls. Confronted by the fact that this virus-induced tumor is endemically present in *R. pipiens* throughout a wide area of this country one might suppose that the virus is present in practically every *pipiens* frog, with tumors developing only in those individuals rendered susceptible by unknown causes. One such cause could be a carcinogenic hydrocarbon which would enable a dormant virus to exert its action. However, no differences were found between injected and control frogs, but any conclusion on the subject is precluded by the fact that according to the present studies the substances used, though very active in inducing malignancy in some mammals, are inactive on frogs.

Summary. The 261 frogs and 18 newts injected with varying amounts of dibenzanthracene, benzpyrene, and methylcholanthrene and kept under different temperatures failed to develop any tumor in

the injected site or to show an increased incidence of the spontaneous Lucké adenocarcinoma of the kidney in *R. pipiens*. The changes induced by the chemicals are mostly of a necrotizing character.

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CONDUCTION VELOCITY AND DIAMETER OF NERVE FIBERS

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Previous studies of the relationship between the size of nerve fibers and the velocity of conduction of impulses have resulted in the bringing forward of three formulations:

1. $V = kD$ (Gasser and Erlanger, 1927; Gasser, 1934)
2. $V = kD^2$ (Blair and Erlanger, 1933)
3. $V = kD^{0.5}$ (Pumphrey and Young, 1938)

At the time at which the present studies were started only the first two proposals had been made, and it was hoped that by the utilization of an experimental method different from those used for the measurements that constitute the basis of the original derivation, new evidence would be acquired which would throw the balance toward one or the other of the two formulas. The method employed depends upon the generally accepted assumption that in any given nerve the largest fiber conducts at the highest velocity. The procedure in accord with the method is to select nerves with different maximal velocities, measure the velocities, and then prepare histological sections of the nerve in which measurements of the diameter of the largest fiber can be made.

A similar method was used by Pumphrey and Young for the small fibers in their nerves. The velocities in the large axons were determined individually and the diameters were measured directly in the fresh state. Squid nerves were used exclusively in their experiments, and this fact must be kept in mind when their data are compared with the data to be presented later, all of which were obtained from medullated vertebrate nerve.

METHOD. In order to obtain valid velocity readings it is essential that the fibers be in the best possible condition. Precaution is especially necessary when small fibers are concerned, as they are more susceptible to injury than large fibers, and if injured they acquire a velocity rating that is too low. The method is a favorable one from the standpoint of the conservation of fibers, as dissection is limited to freeing the nerves of adherent fascia, and it is not necessary to use small strands. Excised nerves of the cat were mounted on silver-silver chloride electrodes in a moist

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chamber, and velocity readings were made as soon as the nerve could be brought into equilibrium with the temperature in the chamber which was maintained at 37.5°C. After the measurements had been made, the nerve was immediately taken out and fixed. Thus distortion of the fibers, which is often seen in nerves that have been subjected to long periods of stimulation, was held to a minimum. Krebs-Ringer solution was used to moisten the nerve during the experiment, and the atmosphere in the observation chamber was 5 per cent CO₂ in O₂.

Measurement of velocity. Condenser discharges controlled by a thyatron were used for stimulation, and the shock strengths were adjusted to give a maximal response of the group of fibers yielding the first elevation in the action potential of the nerve stimulated. Oscillographic records of the action potential were made at five or more distances of conduction, and then the shock-response times were measured on enlarged projections of the records. Because of the utilization period of the shock and the possibility of spread of the stimulus, no single shock-response time can be depended upon for the calculation of the velocity. But the series of times, plotted against the distances, yields a straight line graph the slope of which gives the velocity freed from these sources of error.

High amplification was used for recording. In nerves containing large fibers it was possible, by reducing the strength of shock, to stimulate single fibers and record from them. In view of this fact the conclusion is warranted that the first deviation of the action potential from the baseline in the records made for velocity measurements could be referred to the single largest fiber in the nerve. When small fibers were being studied it was not possible in nerves of the sizes employed to record single spikes with certainty, because of interference by the noise level. Arbitrarily therefore it was assumed, when the maximal velocities were 20 m.p.s. or less, that the first deviation of the action potential should be referred to the three largest fibers instead of to the single largest fiber. Procedure on this basis introduced at most only a small error, since the average diameter of the three largest fibers was ordinarily only a few tenths of a micron less than the diameter of the largest fiber.

Measurement of fiber diameter. When the nerves were removed from the moist chamber they were tied to glass rods to prevent a change in length, and fixed in either 1 per cent osmic acid or 10 per cent formalin. In the latter case they were subsequently stained according to the Kultschitzski technique. The osmic acid method was satisfactory for nerves in which large fibers were to be measured and it had the advantage of being less time-consuming. For nerves presenting small fibers for measurement the second method was preferable. The size measurements, however, were found not to be significantly different when a portion of a nerve prepared in one way was compared with another portion of the same nerve

prepared in the other way. The sections were made after embedding in paraffin, and the diameters of the fibers outside of the myelin sheath were measured with the aid of an ocular micrometer.

Sources of error. 1. *Tapering or branching of the nerve fibers.* The nerves used for embedding were cut into three pieces, and the pieces placed side by side in the paraffin block. Thus each of the finished slides contained in the serial sections samples of the middle segment of the nerve and of the two end segments; and the juxtaposition of the samples made it convenient to determine whether the "largest fiber" tapered or branched in the course of the preparation upon which the velocity measurement had been made. As was to be expected from the straight-line character of the conduction-time conduction-distance curves, no evidence of tapering or branching was found in any of the preparations. It should be stated, however, that the only means of identifying the largest fiber in widely separated nerve regions was by its size, and the measurements reported here establish only the fact that the largest fiber in the peripheral segment of the preparation was not smaller than the largest fiber in the middle and proximal segments.

2. *Random variation of the fiber diameter.* In order to investigate the magnitude of the possible variation in size in the course of the fibers, due to the technique of preparation or other causes, measurements were made of 100 consecutive 6 μ paraffin sections of a saphenous nerve stained with osmic acid. By following one of the small bundles which made up the nerve, it was possible to make sure that the several measurements were all made on the same fiber. The distribution of the measured diameters is shown in figure 1. From the data it can be shown that the standard deviation of a single measurement of a 6.5 μ fiber (modal value) is ± 0.47 μ . As the slides, prepared from the nerves on which the velocity measurements had been made, contained six sections each from three portions of the nerve and as the largest fiber was measured in each of the sections, the error of measurement would be less than that for a single measurement.

If this variation in diameter is brought about by the histological preparation and is not a characteristic of the living fiber, it must occur during the fixation and not in the dehydration process, since Duncan (1934) has shown a comparable diameter alteration along the length of nerve fibers fixed in osmic acid and teased in glycerine.

3. *Differential distortion of large and small fibers.* If nerve fibers of all sizes are not affected in the same manner in the course of histological preparation, the true form of the curve relating velocity and nerve diameter will not be revealed. It was, therefore, necessary to show either that distortion does not occur during the preparation of the nerves, or that if it does occur, it affects fibers of different diameters in a proportional manner.

Donaldson and Hoke (1905) reported that in nine experiments on the

sciatic nerve of the rat there was an average increase in nerve diameter of 0.3 per cent in the course of the steps from the fresh to the paraffin embedded state. They measured the diameter of the entire nerve trunk, as they believed it was impossible to tease out single fibers without stretching them and altering their reactions to histological reagents. Lapique and Desoille (1927) measured the ranges of variation in the diameter of the largest fibers found at the several levels of the frog sciatic nerve. The range at a given level was the same whether the diameters were measured on fresh teased fibers or on the paraffin cross-sections of fixed specimens.

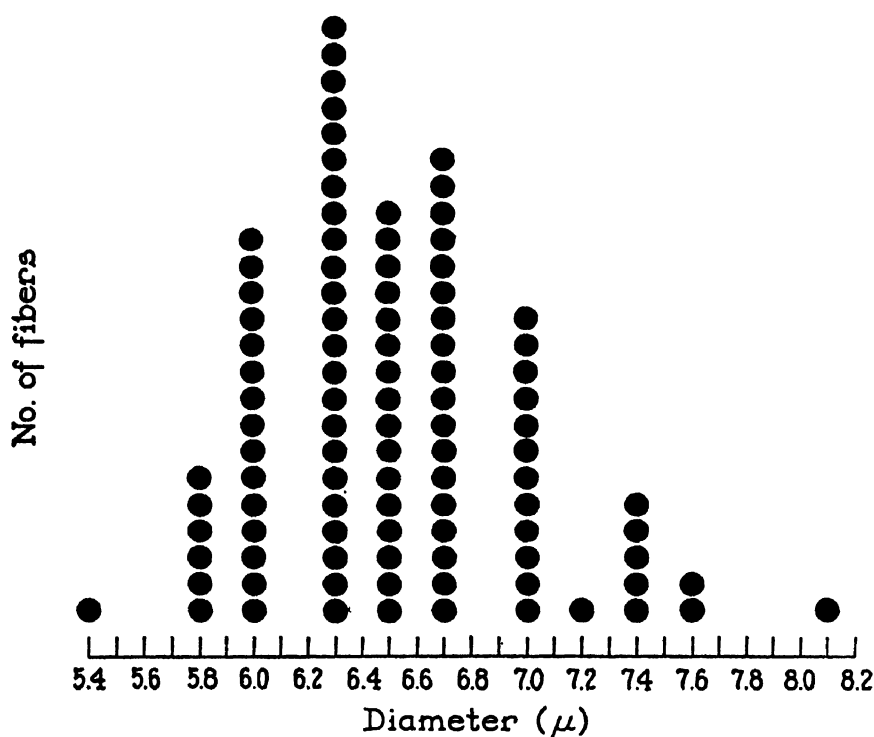


Fig. 1. Distribution of the diameter measurements on a single fiber followed through 100 six-mu serial sections.

Duncan (1934) also examined the question of shrinkage and concluded that the diameters of the living fibers were retained in the mounted cross-sections.

Evidence to the contrary has been presented by Arnell (1936) who measured the fiber diameters in fresh frozen sections and in paraffin sections of the same nerve. Comparison of his tables II and XII shows that a shrinkage of 20 to 30 per cent occurred during the histological preparation. Our experience is in agreement with that of Arnell in showing that shrinkage is produced.

Since the difficulties of dealing with single fibers were recognized, small

spinal root bundles having relatively little connective tissue were used as experimental material. Bundles measuring 0.1 to 0.05 mm. in diameter were attached with ceresin to a glass slide. The slide was ruled at right angles to the course of the nerve, so that measurements could be repeated at exactly the same locus. Diameters at eight points along each bundle were measured by means of a binocular microscope fitted with an ocular micrometer. Fixation in osmic acid caused an average (36 nerve bundles) increase in diameter of 2.2 ± 0.5 per cent. Fixation and dehydration caused an average decrease in the fresh nerve bundle diameter of 23.6 ± 1.2 per cent.

The interpretation of these results was made somewhat ambiguous by the fact that although the spinal root bundles do not have the thick outer connective tissue sheath found in nerve trunks, there is connective tissue between the individual nerve fibers. Doubt, therefore, existed regarding the proportion of the total shrinkage due to connective tissue as compared with that due to nerve fibers.

Inasmuch as fixation in osmic acid did not change the root bundle diameter, it was thought probable that the nerve fiber was not distorted by this treatment. Nerve fibers were, therefore, teased out from a spinal root bundle which had been fixed in osmic acid. Measurements of the nerve diameters were made before and after dehydration. Twenty measurements were made for each diameter and eleven nerve fibers, ranging in size from 22.5 to 10.2 μ , were examined. The shrinkage found varied from 8.2 to 12.9 per cent, with an average value of 10.1 ± 0.16 per cent. There was no correlation between the percentage of shrinkage and the size.

These data considered in relation to the nerve bundle experiments show that a considerable proportion of the shrinkage found during dehydration is due to connective tissue. Shrinkage of the fibers occurs, however, but without indication of its being differential.

Nerves used in the investigation. The maximal velocity of conduction and the diameter of the largest fiber were measured in the following nerves of the cat:

NERVES	MAXIMAL VELOCITIES
	m. p. sec.
Peroneus.....	108-111
Suralis.....	69-86
Saphenous.....	65-82
Vagus.....	67-73
Cervical sympathetic.....	29-56
Hypogastric.....	11-41

RESULTS. The data obtained from these nerves are presented in figure 2. The points seem to fit a straight line more readily than any other

graph. The line which has been drawn is the calculated regression line. It has a slope of 6.0, which means that the velocity in meters per second can be obtained by multiplying the diameter in micra by this factor. The broken lines represent the velocity as a function of the square and the square root of the diameter respectively, assuming that a 13.8 μ fiber conducts at a velocity of 78 meters per second. It is quite apparent that the data are not compatible with either of these two assumptions; and they would be still more incompatible if the square and the square-root

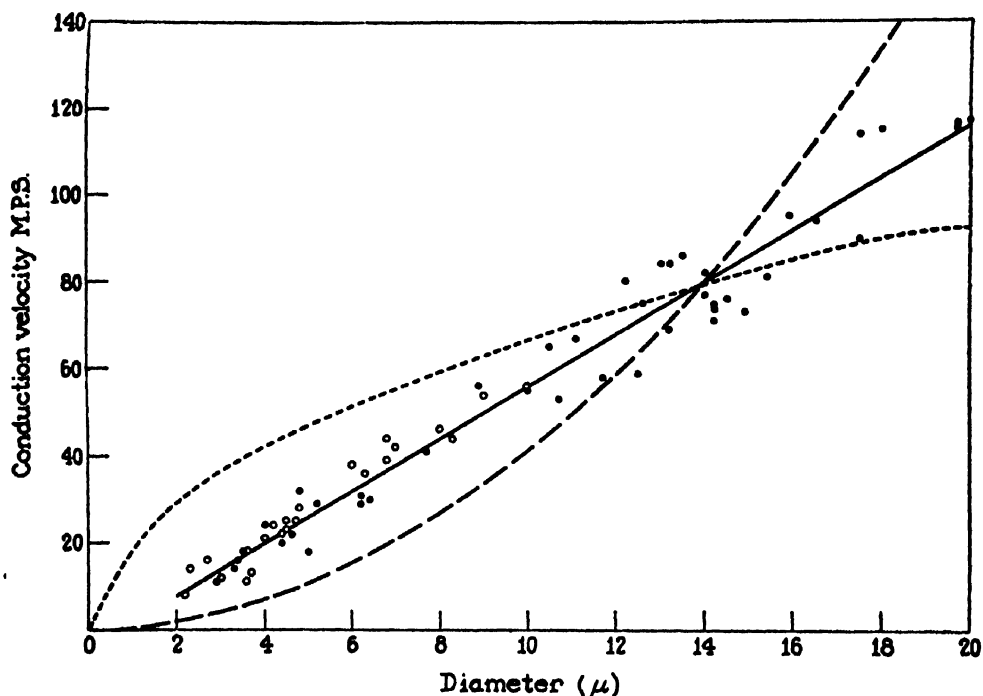


Fig. 2. The fastest conduction rate plotted against the diameter of the largest fiber for nerves of different velocities. The dots are data on adult cats; circles, data on kittens. The dotted and dashed lines represent the velocities calculated as the square root and square functions of the diameter.

curves were drawn to include the points representing the extremes of velocity.

The diameters of the constituent fibers in any particular nerve are known to be smaller in young animals than in the adult (Boycott, 1904; Duncan, 1934). Young animals, therefore, provide an additional source of nerves in which the largest nerve fibers are of small caliber. On these nerves two questions may be studied. Is the conduction velocity in immature nerves proportional to the diameter and is the factor of proportionality the same as that holding for adult fibers?

Both questions may be answered in the affirmative. The circles in

figure 2 present data obtained from the nerve trunks of kittens. It is obvious that they lie very close to the regression line drawn for the cat fibers. The exact slope of the latter is 6.04. The regression line drawn for both the kitten and cat fibers has a slope of 6.00.

DISCUSSION. Correlation of the conduction velocity with the outside diameter of the fiber means that the velocity may be determined by any

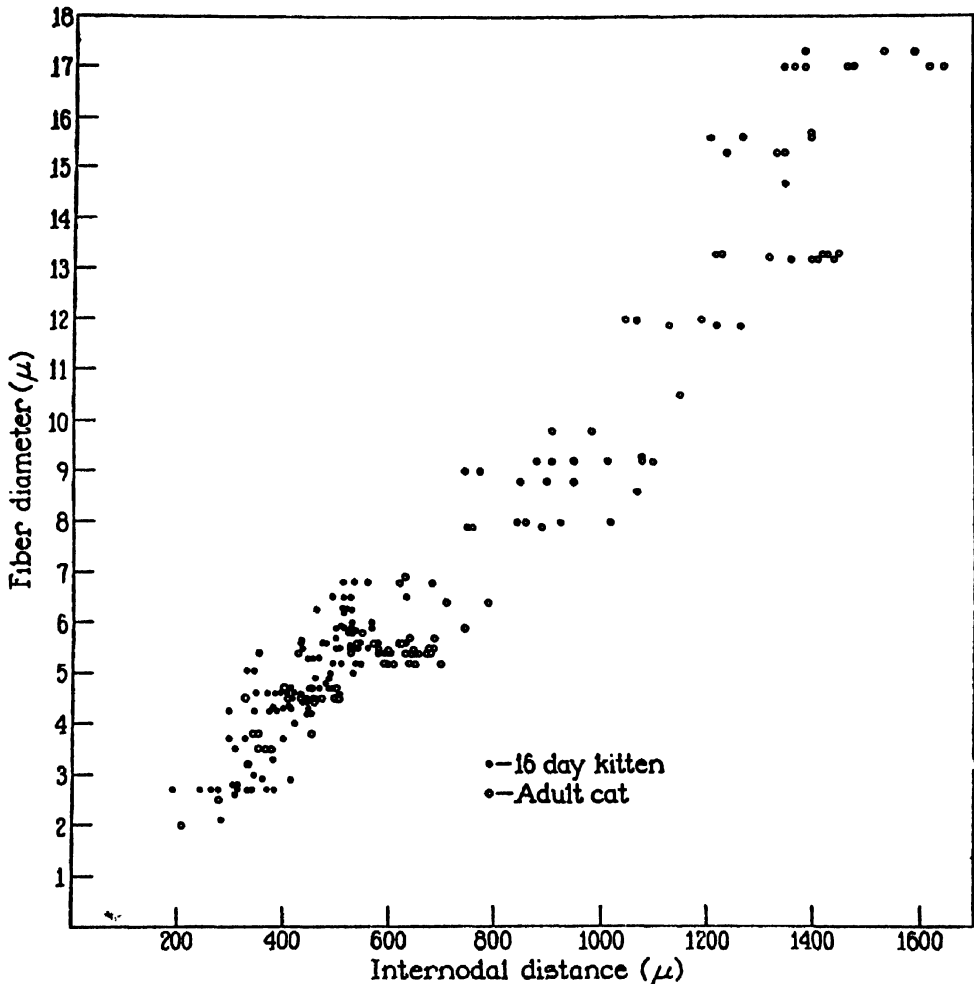


Fig. 3. The internodal distance plotted against nerve fiber diameters of a 16-day-old kitten and an adult cat.

dimension in linear relation to the diameter. The axon diameter is the dimension demanding first consideration. According to Donaldson and Hoke, the ratio of the axon diameter to the total diameter is constant. On the other hand, Arnell (1936) and Schmitt and Baer (1937) find a systematic deviation in the ratio dependent upon the fiber size. The scatter in

the data, however, is too great to permit their use for or against an hypothesis proposing control of the velocity by the axon diameter.

Another dimension to be considered is the length of the internodal segments. The evidence that these lengths are proportional to the fiber diameter has recently been cited by Zotterman (1937). Before the internodal lengths could be considered, however, it would be necessary to show that the lengths are the same in kitten and in cat fibers of the same diameter, inasmuch as kitten and cat fibers of the same size conduct impulses at the same velocity (fig. 2). The point has been especially examined because Boycott in 1904 published data showing that in frog fibers of a given size the internodes are longer in mature frogs than they are in young frogs.

The fibers of the peroneal nerve were teased in a 50 per cent glycerine solution, after fixation with osmic acid according to a method described by Takahashi (1908). The data are presented in figure 3. The dots represent kitten fibers and the circles cat fibers. In the region where the diameters overlap there is very little difference between the internodal lengths for the two ages. The relationship defined by the curve, therefore, is consistent with the substitution of internodal length for the fiber diameter in arguments about the control of velocity.

SUMMARY

The conduction velocities in the fastest fibers in various nerve trunks of the cat and the kitten were correlated with the outside diameters of the largest fibers found in those trunks. The limits of the range of velocities measured were 8 and 117 meters per second.

The best curve relating velocity and diameter is a straight line. It holds equally well in all parts of the range of velocities. Curves drawn in accord with the hypothesis that the velocity varies as the square or square root of the diameter vary widely from the observed points.

Comparative data relating internodal length to fiber diameter are plotted for nerve fibers from cats and kittens.

The author is deeply indebted to Doctor Gasser for suggesting the problem and for his helpful criticism during the course of the work.

It is a pleasure further to acknowledge the advice and assistance of Doctor Grundfest, and to thank Doctor Lorente de N6 for his supervision of the histological technique.

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THE PROPERTIES OF GROWING NERVE FIBERS

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The research here described deals with the changes that take place in the nerve fibers of kittens during the period of growth. Preparations of the saphenous nerve and of the cervical sympathetic trunk were made from kittens ranging in ages from a few days to three months. After the age of three months the properties do not differ essentially from those of corresponding nerves of the adult cat. On these preparations, determinations were made of the velocity of conduction, of the spike duration, the refractory period, and the configuration of the after-potentials. Correlation of the velocity with the diameter of the fibers will be reported in another connection. For the present it may be stated that the velocities of kitten fibers ranging in diameter from 4 μ to 10 μ have been measured. When the velocities and the corresponding diameters are plotted, the points are randomly distributed about a straight line.

In the following description it will be necessary to mention three groups of fibers—A, B, and C. A refers to the myelinated fibers of somatic nerves and to fibers in visceral nerves having similar properties. In sensory nerves the fraction of these A fibers that gives the elevations conducted at about 20 m.p.s. is called delta. B refers to the group labelled B₁ by Bishop and Heinbecker. It is found only in visceral nerves and is made up of fibers having in the adult cat velocities between 15 and 4 meters per second. C refers to the unmyelinated fibers, velocities 2 m.p.s. and slower.

Since, during the period of growth, as much as a sixfold increase takes place in the velocities of the A and B fibers, opportunity is afforded to ascertain the extent to which these groups possess their distinguishing characteristics during the stage in their development at which the velocity of conduction is that of the next slower group. The specific question is asked: Do the spikes, refractory periods and after-potentials in fibers, which in the adult will be called A fibers, have the properties characteristic of A fibers or B fibers, when the fibers conduct impulses at B velocity? And a similar question is asked with respect to immature B fibers conducting at C velocity.

Records were made with a direct-coupled amplifier designed by Doctor

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Toennies. In order to make the spike monophasic, the nerve trunk was in all cases crushed between the two lead electrodes. Despite the exercise of care in removing pieces of connective tissue from the nerve stretch between the electrodes, so as to avoid capacity effects to ground, and in killing the nerves just before the records were taken, a certain amount of diphaseicity is to be observed in some of the records. This difficulty, however, does not interfere to any large extent in the interpretation of the recorded findings. Stimulation was effected by short thyatron discharges applied to the nerve through a transformer. At the conclusion of the experiment certain of the nerves were fixed in osmic acid or formalin and paraffin sections were prepared.

Change of the spike potential with growth. The saphenous nerve. Figure 1 shows spike potentials recorded from saphenous nerves of kittens 4 to 78 days old. Between these ages the velocity of conduction in the fastest fibers increases progressively from 11 to 60 m.p.s. Since all kittens do not grow at an equal rate, better correlation might be expected if some measurement of growth other than age were compared with velocity. The length of the kitten's leg from the head of the femur to the tip of the toes was the measurement selected. In figure 2 these lengths are plotted against the fastest velocities in the corresponding excised saphenous nerve trunks. The relationship as shown here is clearly a linear one.

Two generalizations may be made on the basis of the relationship. First, as far as peripheral nerve is concerned, the movements of the kitten are not slowed because of the decreased conduction rate, since the latter is nicely compensated for by the decreased length of the nerve path. Secondly, it may be inferred that the fibers grow in length and diameter at an approximately equal rate, as the diameter is proportional to the velocity.

In the growth of the saphenous nerve there occurs an early division into the velocity groups that are characteristic of the adult nerve. The record of the 8-day nerve of figure 1 shows the delta group represented as a small elevation conducted at 4 m.p.s. In records taken at various stages of the subsequent growth of the nerve, the delta fibers regularly appear as a distinct velocity group. Frequently there occurs a separation of the faster A fibers into two velocity groups, which gradually merge as the nerve becomes adult.

The cervical sympathetic nerve. Figure 3 shows the potential records at several stages in the growth of that portion of the cervical sympathetic nerve trunk included between the superior and middle cervical ganglia. In order to show fast and slow groups clearly, several sweep speeds and amplifications were employed for recording the 20- and 90-day nerves. The 90-day nerve represents a picture that falls within the range of variation found in the adult nerve: a small fast group of A fibers (32 m.p.s.), a second large group of B fibers (15 m.p.s.), and a slow group of C fibers

(1.7 m.p.s.). In contrast to this picture, the records of the 5- and 8-day nerves show a *single* elevation conducted at velocities typical of adult C fibers. The failure of differentiation into separate velocity groups is

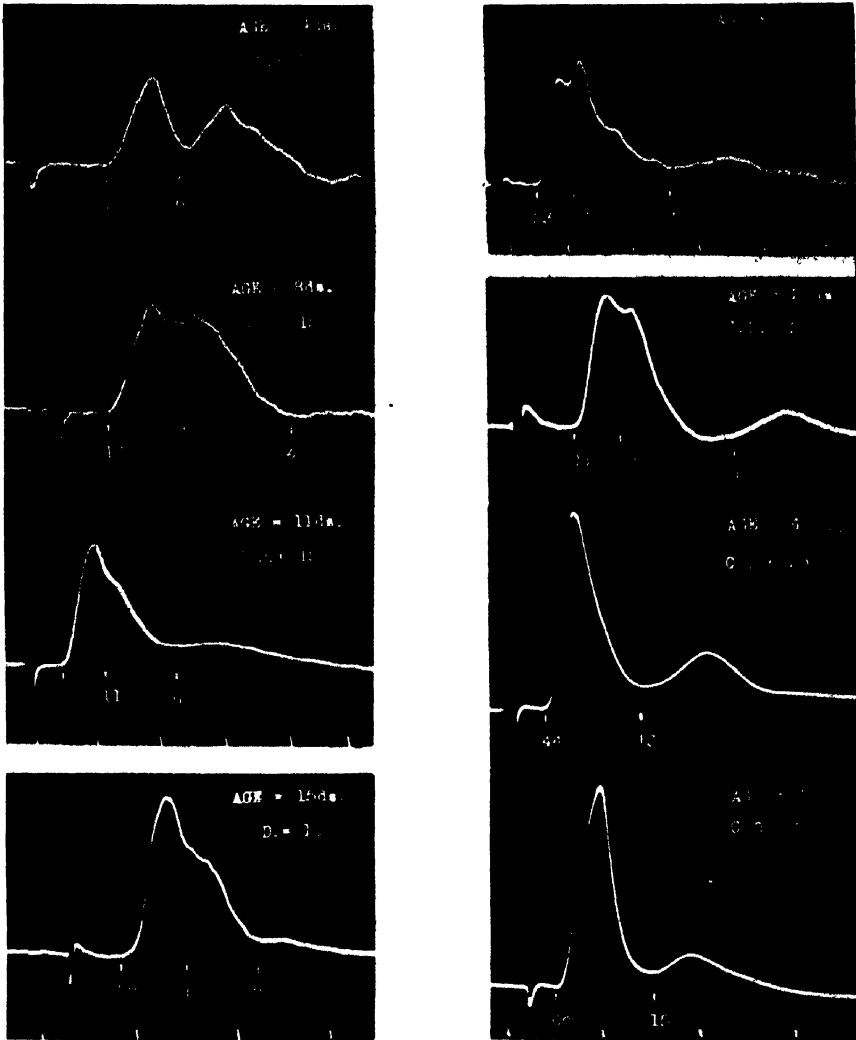


Fig. 1. Changes in the saphenous nerve spike potential during the growth of the cat from 4 to 78 days. The numbers along the bottom of the photographs give the velocities of the denoted groups in meters per second. The pictures are grouped according to the speed of sweep which is noted in msec. at the bottom of the respective groups. The shock was slightly supermaximal.

maintained up to an age of about 12 days, unless depressor fibers are included in the nerve stretch examined. (See table 1, expts. 3, 7 1, 8 1, 10 1.)

Billingsley and Ranson (1918) have described in cats a depressor branch

sometimes observed separate from the vagus in the carotid sheath. Bishop and Heinbecker (1932) regard as afferent vagus fibers the fast group (their S_1), which they usually record ahead of the standard elevations of the cervical sympathetic nerve of the adult cat.

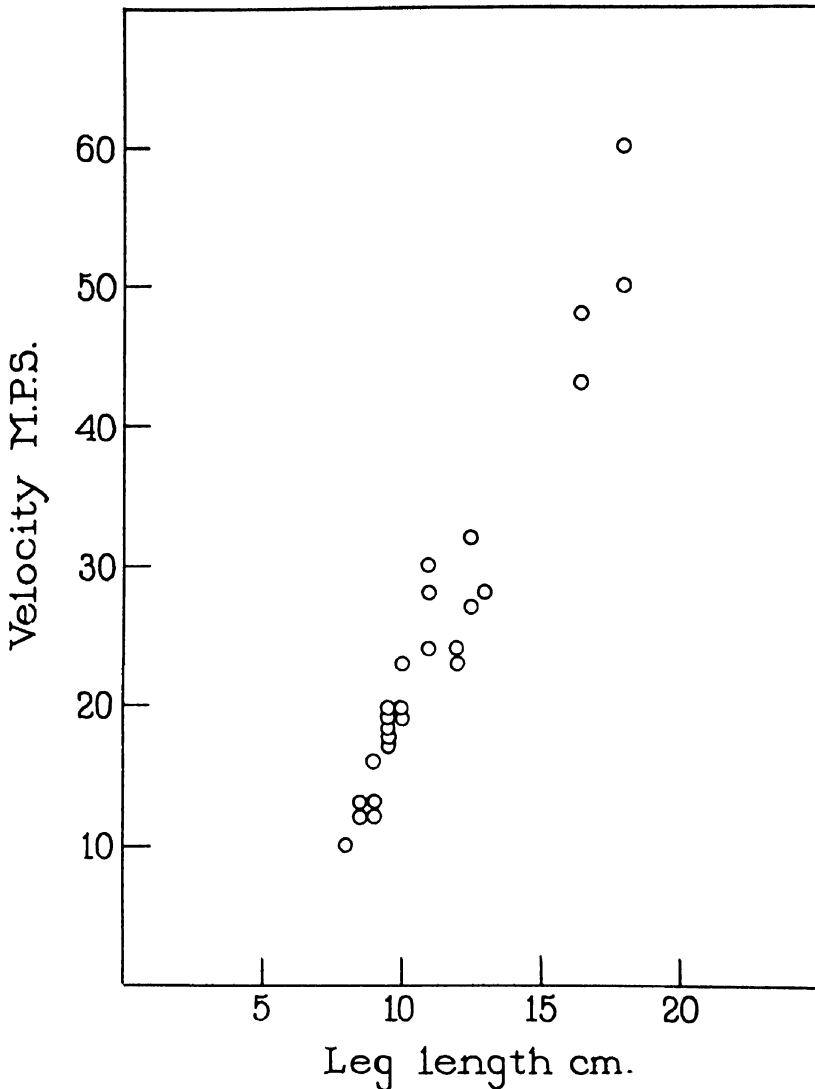


Fig. 2. The relation between the velocity of the fastest fiber in the saphenous nerve and the corresponding length of the kitten's leg, from the head of the femur to the tip of the toes, for leg lengths representing different stages of growth.

In a series of eight experiments performed by the author on adult cats, in a single case, stimulation of the cervical sympathetic nerve brought about a lowering of blood pressure.

In the exceptional cases that are cited above as showing a separate

velocity group preceding the main slow wave, a small branch was found accompanying and frequently joining the cervical sympathetic nerve.

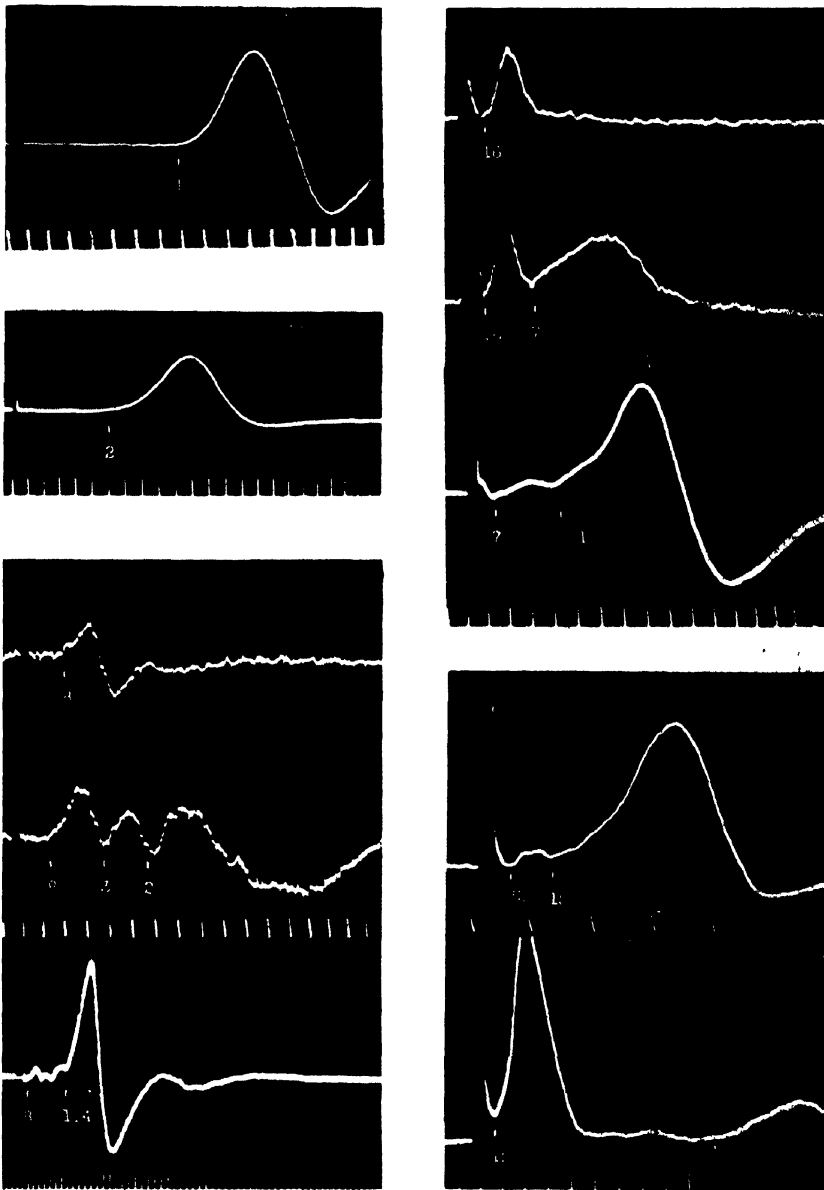


Fig. 3. The cervical sympathetic nerve spike potential at growth stages as labelled. Velocities are noted below the photographs in m.p.s. The 20-day nerves are from the right side (lower set of records) and the left side (upper set) of the same animal. Sweep speeds are in msec.

This branch, when separate, could be dissected back to the nodose ganglion. When the branch and the cervical sympathetic were excised and stimulated

TABLE 1
Growing cervical sympathetic nerve

EXPERIMENT	AGE	VELOCITY GROUPS† AND REFRACTORY PERIODS‡		
	<i>days</i>			
1	4			2.3 (3.0)
2	5			1.3 (2.0)
3	5		7.6* (1.4)	1.6 (3.3)
4 l	5			1.0
4 r	5			1.0 (3.1)
5	8			2.0
6 l	8			1.0
6 r	8			1.8 (3.0)
7 l	8		10.0*	1.6
7 r	8		(1.4)	0.5
8 l	9		6.0*	1.1 (2.6)
8 r	9			1.0 (2.7)
9 l	11		2.2 (2.0)	0.7
9 r	11		2.3 (2.5)	1.5
10 l	11		8.5* (1.1)	1.4 (2.5)
10 r	11			1.3
11 l	12		3.4	1.3
12 l	14		3.3	1.3
12 r	14		3.3	1.1
13	15		1.1 (2.0)	0.6
14 l	16		6.0	1.1
14 r	16		5.7	1.1
15 l	17	15	8.0	2.0 (2.3)
15 r	17		8.6 (1.2)	1.7
16 l	20	16 (1.0)	6.5 (1.5)	2.2
16 r	20		7.6	2.0
17	28		7.5 (1.6)	2.0
18	40	25	10.0	1.7
19 l	60	28	9.0	2.3
19 r	60	18		1.6
20	78	28	12.0 (1.2)	1.7

Experiments marked l and r are nerves from the same animal (l is left side).

* Groups marked in this way are afferent vagus fibers.

† Where clear separation into different velocity groups is found, the fastest velocity of the different groups is noted in m.p.s.

‡ Refractory periods are enclosed in parentheses and are expressed in msec.

separately, the branch potential contained the fast group and the cervical sympathetic only the slow wave.

It is, therefore, concluded that the A fibers of the depressor branch account for the small elevation occasionally found preceding the slow wave in records of 4- to 12-day cervical sympathetic nerves. If at this stage of growth any cervical sympathetic A fibers exist as a separate group, the corresponding spike potential cannot be seen.

Since all cervical sympathetic nerves have a large group of B fibers, it is of interest that no corresponding elevation can be seen in these immature nerves. The possibility that the preganglionic B fibers are not functional, and hence do not contribute to the potential, is negated by the following experiment. An 8-day old kitten was anesthetized with dial and the cervical sympathetic nerve was dissected from the vagus and the depressor trunks. The nerve was cut near the middle cervical ganglion, and the preganglionic fibers leading to the superior ganglion were stimulated. An immediate enlargement of the pupil occurred. The preganglionic nerve was then excised and mounted in a moist chamber for the recording of its action potential. A single elevation, in which the fastest fibers were conducting at 1 m.p.s., was found. If these pupillo-dilator fibers which are functional in kittens are the same as those functioning in the adult cat, they will develop to constitute the fastest part of the B group, as Bishop and Heinbecker (1932) have shown that the pupillary reaction is mediated by fibers the activity of which is manifested in this portion of the action potential.

After the first 12 days, during which the fibers producing the slow wave show no demonstrable increase in velocity (table 1), the action potentials of a small disperse fiber group appear ahead of the main potential elevation. These potentials subsequently increase in size because of the appearance of additional fibers in the group and the growth of those already present. The constituent fibers also increase in velocity. At 20 days the B group is definitely set off from the C group; and ahead of the B group an A group is usually visible.

The saphenous nerve. Effect of growth on the positive after-potential. When the delta component of the A group of fibers first appears, the fastest fibers in the group have a velocity characteristic of B fibers (4-5 m.p.s.). This fact makes it possible to investigate an important point with respect to the after-potentials. At this early stage, are the after-potentials those characteristic of A fibers, in which case one would take them to be determined by the kind of fiber? Or, are they characteristic of B fibers, in which case one would take them to be determined by the size of the fiber?

The method of procedure was to make records of the action potential as excited by two strengths of shocks—the first shock strong enough to

stimulate only the fast group of fibers, and the second shock strong enough to stimulate both groups. A complete separation of the groups could not always be effected, as some late group fibers were stimulated before the quota of early group fibers was complete, but this difficulty was not sufficiently great to destroy the experiments. In these records, the sizes of the after-potentials were measured, directly on the first group and by difference on the second group. The sizes were then compared with the height of the corresponding spike, corrected for temporal dispersion. The correction was made by calculating the height of a spike of 0.5 msec. duration² which would have an area equivalent to the area of the spike complex in the record as measured with a planimeter.

The results of 10 experiments are presented in table 2. These experiments were chosen from a larger number because of the relative absence of diphasicity in the potential records. Diphasicity would upset the after-potential spike-height ratios by making the area of the spike too small and the size of the after-potential too large.

Before examining the table it will be necessary to set forth the dimensions of the positive after-potentials (p.ap.) in A and B fibers. In A fibers the p.ap. ends at 60–80 msec., and its amplitude at the maximum is 0.1–0.4 per cent of the spike height (Gasser and Grundfest, 1936, p. 116). In B fibers the p.ap. ends at 100–150 msec., and its amplitude is 1.5–4.0 per cent of the spike height. (Grundfest, unpublished data.)

The last column in table 2 shows that the p.ap. in immature delta fibers has the properties characteristic of A fibers. The duration is an A duration and the size, while somewhat larger than that associated with the fast A group of the same nerve, is close to A dimensions and far from B dimensions. In one instance only does the size approach a B size (the 8-day nerve) and in this case the duration is distinctly that of an A fiber.

Single spike durations and refractory periods. Table 3 gives the values of single spike durations and refractory periods for the saphenous nerves. The technique employed to record the single spike durations was to use high amplification and to adjust the shock strength at just threshold for the most excitable fibers. As a result, the information obtained concerns only the spike durations of the fastest fiber (strictly the most excitable fiber) in any mixed nerve trunk. It is known that the potential recorded from a nerve diminishes with fiber velocity. Accordingly, a natural

² In this calculation it is assumed that the immature delta fibers have a spike duration of 0.5 msec. Table 3 lists spike durations of 0.60 to 0.70 msec. for A fibers conducting at velocities from 12 to 9 m.p.s. These are average values, however (see following text), and show a range of variation from 0.4 to 0.72 msec. As this evidence for a longer duration is regarded as inconclusive and as the other A spike durations listed in table 3 are independent of conduction velocity, the above assumption may be considered reasonable.

limitation to this method of measuring single spike duration is reached when the amplitude of the recorded spike becomes of the same order as the noise level of the amplifier. The determinations of single spike durations given in table 3 are, therefore, more accurate in the case of the fast than of the slow fibers. The customary procedure was to photograph about 50

TABLE 2

The positive after-potentials of saphenous nerve fibers at various stages of growth

AGE	VELOCITY GROUPS		POSITIVE AFTER-POTENTIAL		
	Weak shock m.p.s.	Strong shock m.p.s.	Duration msec.	Amplitude	
				mv.	Per cent*
<i>days</i>					
8	15		48	12	0.4
		9-5	40	51	0.9
17	18		60	4	0.21
		8.5-5.3	70	7	0.47
19	19		70	8	0.49
		9.3-3.5	85	13	0.45
19	17.5		70	7	0.43
		8.7-5.7	70	7	0.56
20	23-6		60	32	0.50
24	32		62	18	0.20
		8.1-3.0	80	18	0.58
28	32		55	23	0.52
		7.5-3.8	55	16	0.57
28	27		65	19	0.31
		14.3-8.4-4.5	65	17	0.38
40	48		55	14	0.17
		15	67	3	0.10
78 ~	60-15		50	40	0.3

* Amplitude at the maximum calculated as percentage of the height of a 0.5 msec. spike having an area equivalent to that of the recorded spike.

threshold responses. From this group the 10 best examples were selected as those showing the least noise interference at the beginning and the end of the spike. The averages of the durations of these spikes are presented in the table.

The spike durations in table 3 fall within the range of values (0.4-0.5 msec.) that has been determined for the faster fibers of the A group. The

TABLE 3
Growing saphenous nerve

AGE	VELOCITY GROUP	REFRACTORY PERIOD	S.F.S. DURATION	AGE	VELOCITY GROUP	REFRACTORY PERIOD	S.F.S. DURATION
<i>days</i>	<i>m.p.s.</i>	<i>msec.</i>	<i>msec.</i>	<i>days</i>	<i>m.p.s.</i>	<i>msec.</i>	<i>msec.</i>
2	10 3	0.70	0.67	17	28 8 5	1.05	
2	14 3	0.65	0.64	17	19 9	0.56 0.80	
3	9 4	0.60	0.70	17	18 9 6	0.55	
3	14 4	0.60		19	19 7	0.58	
5	12 7 5	0.65	0.60	19	18 9 6	0.58	
5	13 8	0.76		20	23 10 5	0.37	
5	12 8	0.75		27	20 7	0.46	
9	12 4	0.75		27	23 8	0.49	
11	19 7 5	0.51 1.20	0.45	28	28 8	0.53	0.53
11	19 9 5	0.53 1.10	0.55	28	32 8	0.50	
11	12 7 4	0.85		28	27 14 8	0.51	
11	12 4	0.78		40	44 14	0.43	
15	20 7	0.43	0.50	78	60 15	0.40	0.45
17	24 7	0.53	0.45				

first four determinations are made on fibers of low velocity and come within the category referred to above as being less accurate. For this reason some doubt exists that the slightly increased spike durations are significant.

For measurement of the refractory period the conditioning shock was made slightly stronger than that necessary for a maximal nerve response, and the testing shock was increased to three times this value. In a nerve trunk in which the velocities of the component fibers vary regularly, it is clear that the refractory period of only the fastest fibers could be measured. Where there is a clear separation into velocity groups, as in the case of the saphenous nerve, determinations can be made for the fibers heading each group. Thus in table 3, absolutely refractory periods are listed for the delta fibers as well as for the faster A fibers.

The absolutely refractory periods of the most rapidly conducting fibers in growing nerves vary between 0.38 and 0.53 msec. when the velocity is greater than 20 m.p.s. Below 20 m.p.s. in the delta range of adult fibers the refractory period is 0.6 to 0.75 msec. Thus the refractory periods are the same as those of adult fibers conducting at the same velocity. Immature delta fibers have velocities below the range obtaining in the A group of the adult nerve and longer refractory periods. In table 3 it is seen that delta fibers of the 11 and 17-day nerves have absolutely refractory periods about twice that (0.65 msec.) of the fastest adult delta fibers.

The cervical sympathetic nerve. Effect of growth upon the positive after-potential. As immature B fibers conducting at C velocities do not exist as a separate velocity group, and as there is no evidence that they respond to a shock strength lower than that of the C fibers recorded as part of the same potential elevation, it was impossible to obtain an independent record of the after-potential of the immature B fiber.

A maximal shock was, therefore, used and the after-potential recorded following stimulation of both the immature B and C fibers. The proportion of B fibers in the adult cervical sympathetic nerve is high. For example, a fiber count of a cervical sympathetic nerve made by Douglas, Davenport, Heinbecker and Bishop (1934) showed 2005 myelinated fibers, predominately B, as compared with 1663 unmyelinated fibers. It is to be expected that the proportion of B fibers in the young nerves would be the same and that these immature fibers would contribute an important part to the composite after-potential recorded. If the contribution is in accord with the velocity of conduction in the fibers, which has a value typical for adult C fibers, the combined after-potential should resemble in all respects the after-potential of adult mammalian C fibers. Table 4 shows that this is not the case.

The combined potentials have the duration of a C fiber potential with the p.ap. lasting more than a second. But the resemblance breaks down

at that point, as the n.ap., which is regularly found in C fibers, is often either absent (expts. 1, 2 and 5) or reduced (expt. 3) in the combined potential. An explanation of the configuration would be afforded by the assumption that the immature B fiber potentials have a quality resembling that of adult B fibers. The large p.ap. would then synchronize with the n.ap. of the C fibers in the combined potential and tend to cancel it out.

DISCUSSION. The questions that were proposed at the beginning of the paper have been answered in part. A fibers conducting at B velocities display what are essentially A after-potentials. But all that can be said about B fibers conducting at C velocities is that they do not display C after-potentials. This meagre information is not without significance, however, as it indicates that although the configuration of the after-potential in B fibers at the stage at which they conduct impulses at C veloc-

TABLE 4
Cervical sympathetic after-potentials

AGE	VELOCITY RANGE	POSITIVE AFTER-POTENTIAL			NEGATIVE AFTER-POTENTIAL	
		Maximal amplitude	Time to maximum	Total duration	Duration	Maximal amplitude
<i>days</i>		μ	<i>msec.</i>	<i>msec.</i>	<i>msec.</i>	μ
5	1.1-0.7	95	150	1,100		
5	1.1-0.7	102	150	1,100		
5	8*, 1.6-0.7	25	152	1,000	75	8
9	1.0-0.5	48	200	1,200	90	230
11	8*, 1.4-0.3	111	92	900		
17	9-0.4	316	75	400		
60	18-5	42	40	120		

* Small group of depressor fibers.

ity is unknown, it at least has characteristics which are determined to some extent by the kind of fiber and not completely by the velocity. When brought into relation with the fact that the after-potentials in A fibers conducting at B velocities closely resemble those in the adult fibers, the generalization seems justified that the nature of the after-potentials is determined in part at least by the grouping to which the fibers belong.

In contrast to the after-potential, the absolutely refractory period of nerve fibers is not determined by the group to which the fiber belongs. All the measurements of refractory periods that have been made upon kitten fibers are plotted as ordinates in figure 4, with the corresponding velocities as abscissae. In this graph the dots represent A fibers and the circles B fibers. The principal conclusion that can be drawn from these data is that immature A fibers may have refractory periods as long as adult B fibers (1.0-1.2 msec.), and that B fibers conducting at C velocities

may have refractory periods as long as or longer than adult C fibers (1.8-2.0 msec.).

A curve drawn through the points plotted in figure 4 bears a marked resemblance to a comparable curve constructed from the data given by Erlanger (1937, p. 48; see also Blair and Erlanger, 1933) for adult frog

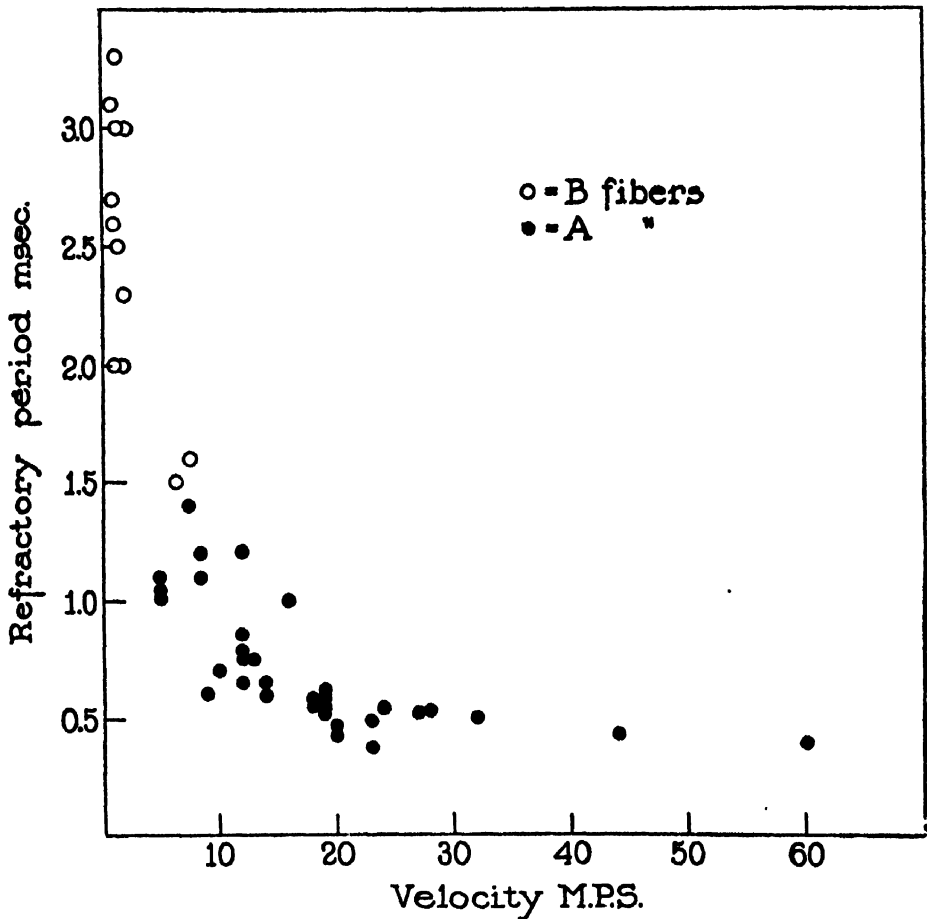


Fig. 4. The refractory periods of immature saphenous and cervical sympathetic nerve fibers plotted against the corresponding velocity of conduction. The dots are data from immature saphenous fibers, the circles from immature cervical sympathetic fibers.

fibers. A single exception to the similarity between the curves is the fact that the kitten fibers show a slight and somewhat questionable increase in refractory period in the range from 60 to 20 m.p.s., while the refractory period of the frog fibers increases more than twofold over the analogous velocity range.

SUMMARY

1. The saphenous and the cervical sympathetic nerves of the cat were studied during their growth.

2. Saphenous nerves of kittens four days old show the A and C elevations in the action potential. As the A fibers grow, their conduction velocities increase. The maximal velocities increase in proportion to the length of the leg.

3. Cervical sympathetic nerves show a single elevation at C velocity in kittens four days old. Differentiation into faster groups starts at about the twelfth day, and the A and B groups are definitely organized at the twentieth day. After the groups appear, the velocities in the constituent fibers increase until adult status is reached.

4. Immature A fibers conducting at B velocities have a positive after-potential with a duration characteristic of A fibers. The amplitude is slightly greater than that characteristic for A, but less than half that characteristic for B. Immature B fibers conducting at C velocity have a positive after-potential differing from that of adult C fibers, but its exact configuration cannot be determined. The after-potentials appear to depend more upon the type of fiber than upon the fiber size.

5. When the absolutely refractory period determinations made upon growing A and B fibers are plotted against the corresponding velocities, the values appear to scatter about a continuous curve irrespective of fiber type. From 60 to approximately 20 m.p.s. the curve displays a slight increase of refractory period from 0.40 to 0.45 msec. At about 20 m.p.s. the increase in refractory period becomes abrupt, so that at a velocity of 10 m.p.s. an average value of about 1 msec. and at 1 m.p.s. an average value of about 2.6 msec. are found.

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THE KINETICS OF PENETRATION

XVIII. ENTRANCE OF WATER INTO IMPALED HALICYSTIS

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When a large plant cell, such as *Valonia macrophysa*, Kütz., or *Halicystis Osterhoutii*, Blinks and Blinks, is impaled on a capillary the restriction on the uptake of substances from the external solution, due to the limited elasticity of the cellulose wall, is removed, and as a result water and electrolyte enter at a much greater rate than into the intact cell. In the case of *Valonia*,¹ for example, the rate of uptake increases about 15-fold. The technique of determining the increase in volume, which is practically equivalent to the increase in the quantity of water in the cell, consists in measuring the rise of liquid in a capillary. This is so simple that it seemed worth while to measure the rate of entrance of water from diluted sea water into individual *Halicystis* cells to determine the absolute rate of water migration in *Halicystis*, and if possible to gain information about the structure of the protoplasm.

The experiments were carried out in Bermuda in the winter of 1936-37 at the Bermuda Biological Station.

EXPERIMENTAL

The method of impaling *Halicystis* cells has been described by Blinks,² and the assembly used when the rise of liquid in the capillary is to be measured has already been discussed by us.¹ In the present case the experiments were carried out at 17°C. $\pm 1^\circ\text{C}$. which was the temperature of the sea water flowing through the tray in which the bottles containing the cells were immersed. Before impalement the volume of each cell was determined individually by the method pre-

¹ Jacques, A. G., *J. Gen. Physiol.*, 1938-39, 22, 147.

² Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 223.

viously described.³ After impalement each cell was allowed to stand in normal sea water at least 48 hours, during which time the rate of increase of the volume of the sap was measured, to make sure that it followed an approximately linear course, since from other experiments, to be described in another paper, this appears to be the regular behavior in normal sea water.

The sea waters to which the cells were exposed ranged from 90 per cent down to 30 per cent and the length of exposure was such that the amount of water entering would not dilute the sap by as much as 10 per cent: usually the calculated dilution was not more than 5 per cent.⁴ Each cell was exposed to all the diluted sea waters and between exposures each was allowed to stand at least 24 hours in normal sea water in order to recover from the dilution of the sap. During this time the sap volume decreased somewhat, as shown by the fall of liquid in the capillary. But within a comparatively short time the entrance of electrolytes and water was resumed and when it attained a linear rate about the same as that observed before the cell was exposed to dilute sea water, recovery was considered to be complete.

RESULTS

The results of a typical experiment are given in Fig. 1. This shows the rate of increase in volume of the sap of a single cell on exposure to dilute sea waters. The increase in volume has been calculated from the formula

$$\Delta V = H\pi r^2$$

where H is the increase in the height of liquid in the capillary which was measured by means of a micrometer caliper reading to 0.02 mm., and r is the radius of the capillary obtained by direct microscopic measurement of the diameter of the capillary, by means of a micrometer ocular.

Without any assumptions as to the nature of the protoplasm, we may assume that

$$\frac{dQ}{dt} = \frac{k_1 A}{h} (N_o^{\text{H}_2\text{O}} - N_i^{\text{H}_2\text{O}}) \quad (1)$$

³ Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, 15, 537.

⁴ The initial cell volume and the increase in volume being known it was possible to calculate the dilution if no electrolyte were lost by the cell. Probably, however, a little electrolyte was lost but the amount was not large even when the sea water was diluted to 30 per cent. Some data are given on this point farther on in the paper.

where $N^{\text{H}_2\text{O}}$ is the concentration of water on the mole fraction basis, A is the surface area, and h the thickness of the membrane, and o and i refer to the sea water and sap respectively.

If we assume that there are non-aqueous surface layers at the sap-protoplasm and sea water-protoplasm interfaces we must take into account the process of solution of water in the non-aqueous layers.

From Osterhout's treatment of the guaiacol model we assume that at each interface there are in contact unstirred layers, one in water and the other in the protoplasm, and that at the immediate interface there are thin regions in each layer where all the molecular species in both phases are in equilibrium across the boundary. For any species, say r , we can write for the sea water-protoplasm interface

$$S'_{\text{eop}} N'_{\text{eop}} = N'_{\text{epo}}$$

and for the sap-protoplasm interface

$$S'_{\text{eip}} N'_{\text{eip}} = N_{\text{epi}}$$

where eop and epo refer to the portions of the unstirred layers, in sea water and protoplasm respectively, where the equilibrium prevails, and S' is the partition coefficient of r . If we are dealing with water which is present in much greater abundance than any other species we may assume that there is no gradient of water across the unstirred layers so that

$$N^{\text{H}_2\text{O}}_{\text{eop}} = N^{\text{H}_2\text{O}}_o \quad \text{and} \quad N^{\text{H}_2\text{O}}_{\text{eip}} = N^{\text{H}_2\text{O}}_i$$

Hence the rate of entrance of water is equal to the flux of water across the non-aqueous protoplasmic layers, and if we can treat these as a single thin layer, then the flux is given by

$$\frac{dQ}{dt} = \frac{k_1 A}{h} (N^{\text{H}_2\text{O}}_{\text{epo}} - N^{\text{H}_2\text{O}}_{\text{epi}}) \quad (2)$$

or

$$\frac{dQ}{dt} = \frac{k_1 A}{h} (S_{\text{eop}} N^{\text{H}_2\text{O}}_o - S_{\text{eip}} N^{\text{H}_2\text{O}}_i) \quad (3)$$

If the water added to the sap mixes without expansion or contraction of volume we can write for the sap $\frac{dV}{dt}$ for $\frac{dQ}{dt}$.

Assuming that the solutions are ideal dilute ones, we may now apply the gas laws, whence

$$P_e V_e = P_t V_t = P_s V_s = \text{a constant} \quad (4)$$

where P is the osmotic pressure, V is the volume of the sap, e refers to conditions at the theoretical⁵ end of the experiment when sap and sea water are in osmotic equilibrium, t to any time t during the exposure, and s to the beginning of the exposure (zero time).

But

$$N_{\text{H}_2\text{O}} = 1 - \Sigma N^{\text{sol.}}$$

where the index "sol." means solute.

Now for 1000 ml. of a dilute solution of an electrolyte, we may say that

$$P = \frac{i m R T}{V} = \frac{i n^{\text{sol.}} R T}{1000}$$

where m is the molar concentration of the solute, V is the volume of the solution, assumed to be equal to the volume of the solvent (water) alone, i is the van't Hoff coefficient, and $n^{\text{sol.}}$ is the number of moles of solute. Or

$$P = \frac{i}{V_m} \frac{n^{\text{sol.}} R T}{n_{\text{H}_2\text{O}}}$$

where V_m is the molal volume of the solvent = $1000 \div 55.5 \simeq 18.0$, and $n_{\text{H}_2\text{O}}$ is the number of moles of water.

But in an aqueous solution in which $n_{\text{H}_2\text{O}}$ is large compared with $n^{\text{sol.}}$ we can write

$$\Sigma N^{\text{sol.}} = \frac{\Sigma n^{\text{sol.}}}{n_{\text{H}_2\text{O}}}$$

whence

$$P = \frac{i R T \Sigma N^{\text{sol.}}}{V_m}$$

⁵ Theoretical in the sense that the experiments were never allowed to go to equilibrium and it is probable that if they had the measured V_e would have been smaller than the theoretical V_e for the reason that as the exposure progressed some electrolyte would have been lost by the cell.

From this point on $\frac{iRT}{V_m} = \omega$ another constant.⁶

Then

$$\frac{dV}{dt} = \frac{k_1 A}{h} \left\{ S_{osp} \left(1 - \frac{P_s}{\omega} \right) - S_{aip} \left(1 - \frac{P_t}{\omega} \right) \right\} \quad (5)$$

dividing through by S_{aip} and rearranging we get

$$\frac{dV}{dt} = \frac{k_1 A S_{aip}}{h} \left\{ b - 1 + \frac{P_t}{\omega} - \frac{P_s}{\omega} \right\} \quad (6)$$

where b equals the ratio $S_{osp} \div S_{aip}$. Or substituting for P

$$\frac{dV}{dt} = \frac{k_1 A S_{aip}}{h} \left\{ b - 1 + \frac{\eta V_s - \eta b V_t}{V_s V_t} \right\} \quad (7)$$

where $\eta = P_s V_s \div \omega$. Putting $b = 1$ and integrating gives

$$\frac{k_1 A S_{aip} t}{h} = \frac{V_s \omega}{P_s V_s} \left[V_s - V_t + 2.3 V_s \log \frac{V_s - V_s}{V_s - V_t} \right] \quad (8)$$

Putting $k_1 S_{aip} \div h = k$ the equation becomes

$$\frac{k}{\omega} = \frac{V_s}{i P_s V_s A} \left[V_s - V_t + 2.3 V_s \log \frac{V_s - V_s}{V_s - V_t} \right] \quad (9)$$

which is practically identical with that of Northrop.⁷ It differs only in containing S_{aip} and in that the term ω is explicit. ω is the proportionality constant relating osmotic pressure to the concentration of the solution expressed as mole fraction of solute. Northrop's equation was obtained by integrating

$$\frac{dV}{dt} = \frac{k A P_s V_s}{h} \left(\frac{1}{V_t} - \frac{1}{V_s} \right) \quad (10)$$

Equation (9) has been used in the present work to calculate permeability constants of water entrance. In these calculations V_s was

$$^6 \quad \omega = \frac{iRT}{V_m} = \frac{1.8 \times 1.9885 \times 4.015 \times 10^{-2} \times 290.6}{0.018} = 2320 \text{ atmospheres}$$

i is taken as 1.8 which is the approximate value for the 0.61 M sodium chloride solution, to which *Halicystis* sap very nearly corresponds.

⁷ Northrop, J. H., *J. Gen. Physiol.*, 1927-28, 11, 43.

obtained directly by a displacement measurement of water, $V_i = V_s + \Delta V$. ΔV was measured⁸ directly from the rise in the capillary. A was calculated from V_s on the assumption that the cell is spherical and since care was taken to use cells as nearly spherical as possible this assumption is a reasonably good one. P_s was calculated from the average freezing point lowering of *Halicystis* sap (which is 2.059°C.) for the relationship

$$P = \frac{L\rho\Delta T}{T}$$

where L is the latent heat of fusion of the solvent, ρ is the density of the solution,⁸ ΔT is the lowering of the freezing point, and T is the freezing point of the solution. Taking $L = 80$ cal. and 1 gm. cal. = 41.3 cm.³ atmospheres,

$$P_s = \frac{80 \times 1.026 \times 2.059^\circ}{271.04^\circ} = 0.6147 \text{ gm. cal./cm.}^3 = 25.39 \text{ atmospheres}$$

V_s has been calculated from V_i on the assumption that $P_s = P_o$, where P_o is the osmotic pressure of the sea water to which the cell is exposed. Then

$$V_s = \frac{V_i P_s}{P_o}$$

Assuming that the van't Hoff coefficient remains constant over the range of dilute sea waters employed,

$$V_s = \frac{V_i C_s}{C_o}$$

In this calculation $C_s = 100$ per cent and $C_o =$ per cent composition of the dilute sea water.

Values for the constant k/ω are given in Table I: these are calculated from the data of Fig. 1. Neglecting the first and second minutes, which show large trendless irregularities, it will be observed that the "constant" remains reasonably constant in each sea water dilution for

⁸ We have assumed that *Halicystis* sap is a 0.6 M solution of sodium chloride, the density of which at 0°C. is according to interpolation from Baxter and Wallace's data 1.026. This value of ρ has been used in our calculation.

at least the first 10 minutes of the exposure. Thereafter it decreases fairly regularly in most cases, and the decline is too great to be merely the result of experimental error. To save space, values after the first 10 minutes have been omitted but the value from the last reading and the time are given.

TABLE I

Constants* ($k/\omega \times 10^5$)

$$k/\omega = \frac{V_s}{V_s P_s A t} \left(V_s - V_t + V_s 2.3 \log \frac{V_s - V_s}{V_s - V_t} \right)$$

$$V_s = 0.423 \text{ cm.}^3 \quad P_s = 25.39 \text{ atm.} \quad A = 2.724 \text{ cm.}^2$$

Time <i>min.</i>	Per cent concentration of sea water						
	30	40	50	67	75	83	90
1	1.42	2.91	2.18	2.27	3.53	2.52	3.31
2	2.91	2.52	3.01	2.89	3.58	3.42	2.91
3	2.00	2.49	2.64	3.43	3.18	3.42	2.72
4	2.01	2.37	2.95	3.02	2.87	3.20	—
5	2.17	2.36	2.97	3.21	2.74	2.91	3.32
6	2.13	2.33	2.84	3.00	2.76	3.31	3.15
7	2.01	2.30	2.81	2.94	2.76	3.20	2.94
8	2.00	2.39	2.77	2.96	2.78	3.41	3.14
9	1.92	2.19	2.71	3.01	2.76	3.22	3.12
10	2.03	2.31	2.66	3.09	2.74	3.21	3.05
	1.88	2.03	2.32	2.53	2.28	2.85	2.72
	†(20)	(20)	(35)	(60)	(75)	(90)	(90)
Average k/ω between 3 to 10 minutes	2.04	2.34	2.79	3.08	2.82	3.24	3.06

* $k = k_1 S_{a,p} \div h$, where k_1 is the true permeability constant, *i.e.* the number of cubic centimeters of water entering the cell per minute, through 1 cm.² of a surface layer 1 mm. thick under a difference of osmotic pressure of 1 atmosphere. ~

† Figures in parentheses are times in minutes at which last readings were made.

Neglecting the last part of each run and omitting the first 2 minutes, which show the large deviations, not unusual at the start of kinetic processes, we have calculated the average value of k/ω (see page 287) for the period between 2 and 10 minutes. The results are given in Table I and have been plotted in Fig. 2 which contains, in addition,

the values of k/ω against per cent concentration of sea water for other cells for which sufficient data are available, and an average curve for all points common to these cells. Although there are rather large deviations, each curve and the average curve show unmistakably an abrupt drop as the concentration of sea water drops below 50 per cent. This is clearly brought out in Fig. 1, where the curves for 40 and 30 per cent sea water coincide with each other and approximately with the 50 per cent curve. Above 50 per cent k/ω is either constant or perhaps it drops very slowly.

We must now consider whether the equation here used for the calculation of k/ω , which indicates that it suffers some change with time at constant external sea water concentration, and also some change with sea water concentration, really represents accurately the behavior of water.

In his discussion of the diffusion of water into *Arbacia* eggs, Northrop pointed out that it might be necessary to take into account (a) the increase in the surface and decrease in the thickness of the membrane, or (b) if a pore system is in question the increase in the diameter of the pores and the decrease in their length, as the cell increases in volume.

For both cases the same equation was derived and integrated, and a somewhat similar equation was obtained by Lucké, Hartline, and McCutcheon,⁹ also for the swelling and shrinkage of *Arbacia* eggs. Both their equation and Northrop's new equation, gave better constants than the original Northrop equation. Furthermore, k/ω calculated on the basis of Northrop's original equation decreased with the decrease in concentration of the sea water. This effect disappeared when the new equation, taking into account the changes in the dimensions of the membrane, was employed. Because in our work the value of k/ω calculated from Northrop's original equation decreased rather abruptly when the sea water concentration was 50 per cent or less, it seemed fair to inquire whether we should attempt to apply Northrop's new equation to our data. On reflection this seemed to be an unjustified step, since in the case of an impaled cell there is

⁹ Lucké, B., Hartline, H. K., and McCutcheon, M., *J. Gen. Physiol.*, 1930-31, 14, 405.

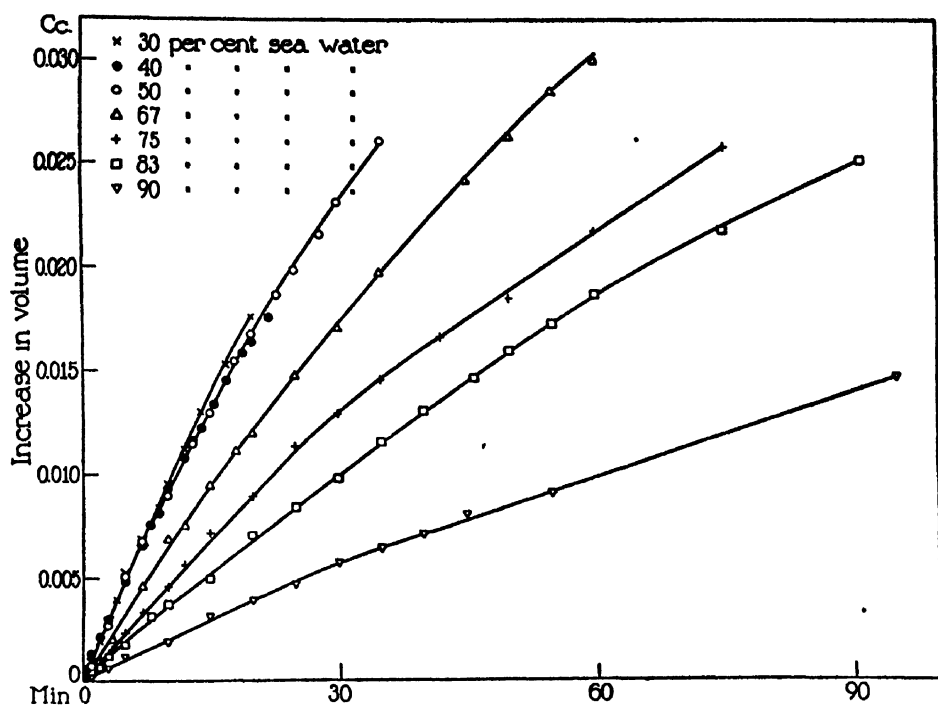


FIG. 1. Rate of entrance of water into a single cell of *Halicystis Osterhoutii* from dilute sea water. The curves are drawn free-hand to give an approximate fit.

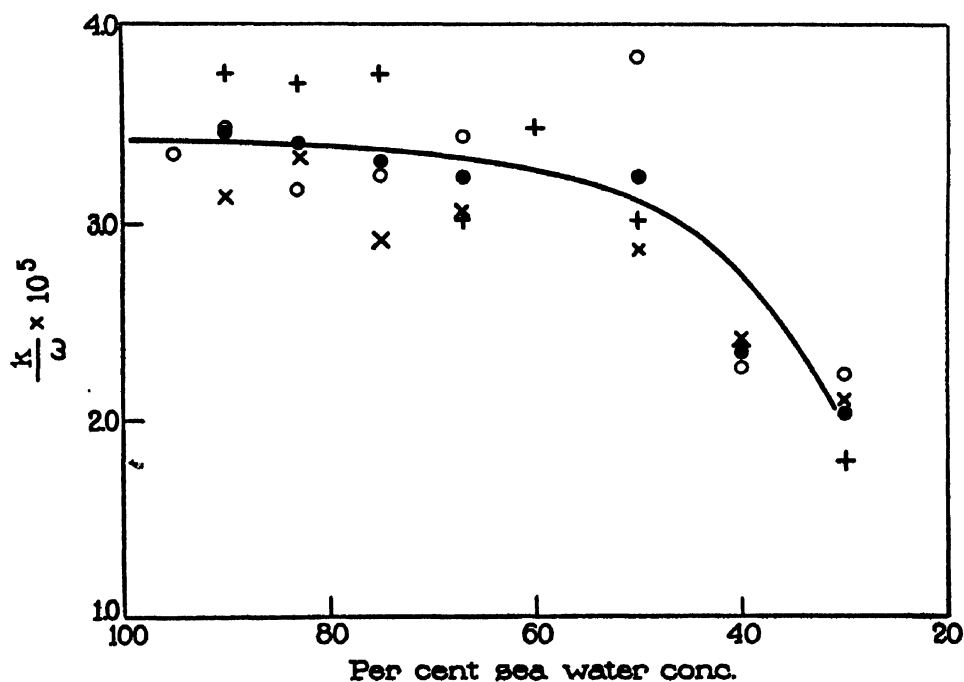


FIG. 2. Velocity constants plotted against per cent concentration of sea water. The curve is drawn free-hand to give an approximate fit.

no evidence that the surface area changes at all.¹⁰ Indeed this is an outstanding advantage of this type of experiment. The equation was therefore not used and we are satisfied with the conclusion on the basis of the old equation that the absolute rate does decrease abruptly somewhere around 50 per cent sea water concentration.

If it were possible to apply corrections for the deviations of the gas laws, which apply strictly to ideal dilute solutions only, some rectification of k/ω as a function of time might be possible. Unfortunately not much progress which is thermodynamically sound can be made in this direction. But it is possible to show that probably any rectification which would be achieved by correcting for deviation from ideal will be negligible.

Strictly the relationship of equation (4) should be

$$\frac{P_s V_s}{\phi_s} = \frac{P_t V_t}{\phi_t} = \frac{P_o V_o}{\phi_o} = \text{constant} \quad (4a)$$

where ϕ is the osmotic coefficient of Bjerrum.¹¹ This leads to

$$\frac{dV}{dt} = \frac{kAP_s V_s}{h\phi_s} \left(\frac{\phi_t}{V_i} - \frac{\phi_o}{V_o} \right) \quad (4b)$$

for equation (10).

This equation cannot be integrated readily because ϕ in concentrated solution is not simply related to the concentration or the osmotic pressure of the solution.¹²

¹⁰ It is possible that in the course of an exposure of a cell to dilute sea water, the rise of the liquid in the capillary might cause actual stretching of the membrane. But since this rise was never more than 2 cm. this effect may be neglected.

¹¹ ϕ is defined by $\frac{P_{\text{ideal}} - P_{\text{real}}}{P_{\text{ideal}}} = 1 - \phi$. It is related to the j function used by Lewis and Randall (Lewis, G. N., and Randall, M., *Thermodynamics*, New York, McGraw-Hill Book Company, Inc., 1923, 286) $1 - \phi = j$.

¹² In dilute solutions of uni-univalent electrolytes, according to Lewis and Linhart (Lewis, G. N., and Linhart, G. A., *J. Am. Chem. Soc.*, 1919, **41**, 1951) $j = \beta m^{\frac{1}{2}}$, where m is concentration in molal terms and β is a constant. Unfortunately as m increases even in pure solution of uni-univalent electrolytes, the relationship between j and m becomes very complex. Scatchard (Scatchard, G., *Chem. Rev.*, 1936, **19**, 309) following Debye has derived quite different equations relating ϕ and the ionic strength of concentrated solutions either pure or mixed, but his equations cannot be applied to the present case, since they introduce new variables.

About the best we can do is to show that probably the osmotic coefficients over the range of sea water and sap concentrations used are so little different from each other that the correction can be neglected.

If we assume that the sap of *Halicystis* is approximately 0.6 M NaCl solution¹³ the osmotic coefficient, according to Scatchard and Prentiss¹⁴ freezing point measurements, is 0.91. And if in the course of its exposure it is diluted to the theoretical limit, so that $P_e = P_s$, then in 30 per cent sea water its concentration should drop to about 0.18 M at which the osmotic coefficient is approximately 0.92. This is the maximum sweep which would occur in the experimental work. Accordingly we are justified in assuming that ϕ is a constant in the present case, and as such it cancels out in equation (4 b).

The decrease of k/ω with time might be due to the slow loss of electrolyte by the cell, since the protoplasmic membrane is clearly not a true semi-permeable membrane. The movement of electrolyte may be so slow in comparison with the movement of water that at the start (within the limits of experimental error) the cell appears to act as a true osmometer and a reasonably accurate value for the permeability constant is obtained. Only after a comparatively long time does the effect of the loss of electrolyte appear. In the present experiments a few tests of the loss of electrolyte have been made by exposing cells to dilute sea water and then comparing the calculated concentration of halide with the actual concentration found from analysis.

In making these calculations the concentration of halide of impaled cells 24 hours after exposure to normal sea water was taken as 0.5975 M. This is the average of 4 cells. But the individual measurements varied from 0.6020 M to 0.5900 M. Because of this variation the calculated values are somewhat uncertain, but in every case they

¹³ The composition of *Halicystis* sap, according to Blinks and Jacques (Blinks, L. R., and Jacques, A. G., *J. Gen. Physiol.*, 1929-30, 13, 733) is

Chloride	0.6028 moles per liter
Sodium	0.5570 " " "
Potassium	0.0064 " " "
Calcium	0.0080 " " "
Magnesium	0.0167 " " "
Sulfate	Trace
	(0.5881 " " "
Total cations	0.603 gm. equivalents per liter

¹⁴ Scatchard, G., and Prentiss, S. S., *J. Am. Chem. Soc.*, 1933, 55, 4355.

were higher than the values actually determined, which suggests that some electrolyte had been lost. For example, a cell in 30 per cent sea water for 30 minutes increased its sap volume in such a way that $\frac{V_o}{V_o + \Delta V}$ of 0.5975 M (the calculated value) was equal to 0.5735 M, but the actual value was 0.5520 M. The calculated value may be a little high for the reason that V_o , the original cell volume, was that of the intact cell but since the cell contracts on impalement, by spurting, a smaller V_o should be used in the calculation. It is estimated that the cell volume may shrink about 20 per cent in this way, at the start, and if we apply a 20 per cent correction¹⁵ to V_o , the "corrected" calculated value of the halide concentration becomes 0.5681 M. The figures therefore indicate that there is some loss of electrolyte and that it is small. But it may possibly account for the decrease in k/ω with time. But, on the other hand, the large drop in the "constant" when the external sea water concentration is below 50 per cent clearly cannot be accounted for in this way, since the rate of loss of electrolyte is too slow.

If the water passes through the protoplasm by diffusing in pores we should expect the rate of increase of volume dV/dt to increase steadily as the concentration gradient of the water between sea water and sap increases. That is k/ω should remain constant. But, of course, if for any reason, as the sea water is made more dilute, k increases or the radius of the pores decreases,¹⁶ then k/ω should decrease. But the only probable way in which either k or the pore radius can change is by an increase in the volume of the non-aqueous part of the protoplasm due to the taking up of more water as the water concentration of the sea water increases.

But as water is soluble in the non-aqueous protoplasm we can

¹⁵ It may be suggested that this decrease in volume should be taken into account in estimating k/ω . This is strictly true but since V_o is estimated from V , the discrepancy due to neglecting this loss is partially wiped out. For example, if we take the case of the cell in 30 per cent sea water. Instead of taking $V_o = 0.423$ cc. we take it as 0.3384; i.e., 20 per cent less. The k/ω at 3, 5, and 10 minutes are respectively 0.00238, 0.00255, and 0.00237. Thus while we get slightly different values for the "constant" these also show no trend.

¹⁶ If diffusion is in pores A is equivalent to the area of the pores, rather than the total area of the membrane.

readily conceive that it is transferred between the sap and sea water without passing through pores. The decrease in the value of the constant might then be accounted for on the assumption that the partition coefficients change.

SUMMARY

The rate of entrance of water into impaled cells of *Halicystis Osterhoutii*, Blinks and Blinks, has been determined directly by measurements of the rise of sap in a capillary for dilute sea waters (containing between 90 and 30 per cent sea water).

The velocity constant remains reasonably constant down to 50 per cent sea water but it decreases markedly in lower concentrations.

THE KINETICS OF PENETRATION

XIX. ENTRANCE OF ELECTROLYTES AND OF WATER INTO IMPAIRED HALICYSTIS

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In a previous paper¹ it was shown that when cells of *Valonia macrophysa*, Kütz., are impaled on a capillary and immersed in normal sea water the rate of entrance of water and of electrolyte is about 15 times as great as with intact cells. In the present paper it is shown that similar results are obtained with impaled *Halicystis Osterhoutii* (Blinks and Blinks).

The experiments were carried out in Bermuda at the Bermuda Biological Station in the winter of 1936-37.

EXPERIMENTAL

The setup for the measurement of the increase in volume for single impaled cells has already been described in a previous paper,¹ and the same technique was followed here. Briefly it consists in impaling a cell on a very thin-walled capillary drawn on the end of a tube of capillary bore, exposing it to normal sea water, and determining the rate at which the volume increases from the increase in height of the sap in the capillary tube. The rise in height was determined by means of a micrometer caliper reading to 0.02 mm. and the volume was calculated from the formula

$$V = \pi r^2 h$$

where r is the radius of the capillary, and h is the capillary rise. The radius for each capillary tube was determined by direct measurement of the diameter with a microscope fitted with an ocular micrometer. The temperature of the sea water during the exposure was maintained at 17°C. \pm 1°C. by immersing the bottles

¹ Jacques, A. G., *J. Gen. Physiol.*, 1938-39, 22, 147.

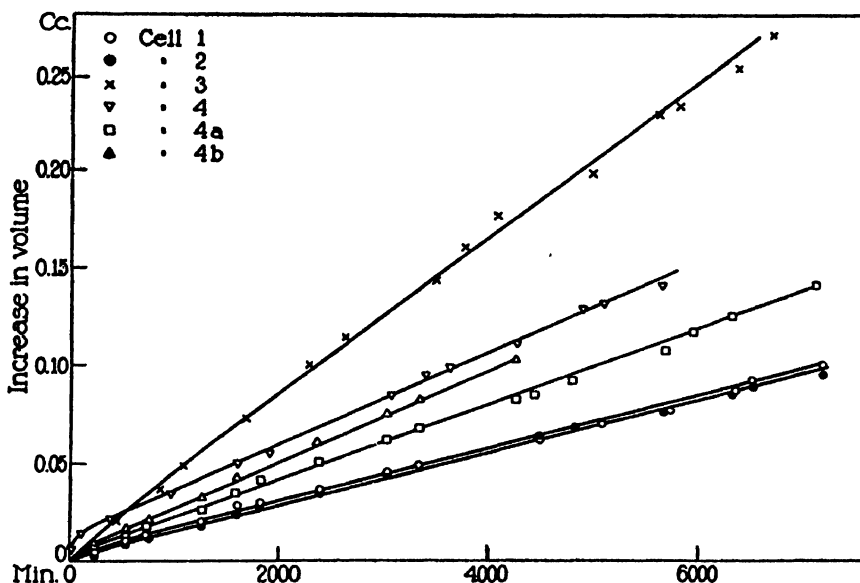


FIG. 1. Rate of increase of sap volume of impaled *Halicystis* in normal light (i.e. natural succession of daylight and darkness). The curve for Cell 4 (∇) starts immediately after impalement; the other curves start 24 hours or more after impalement.

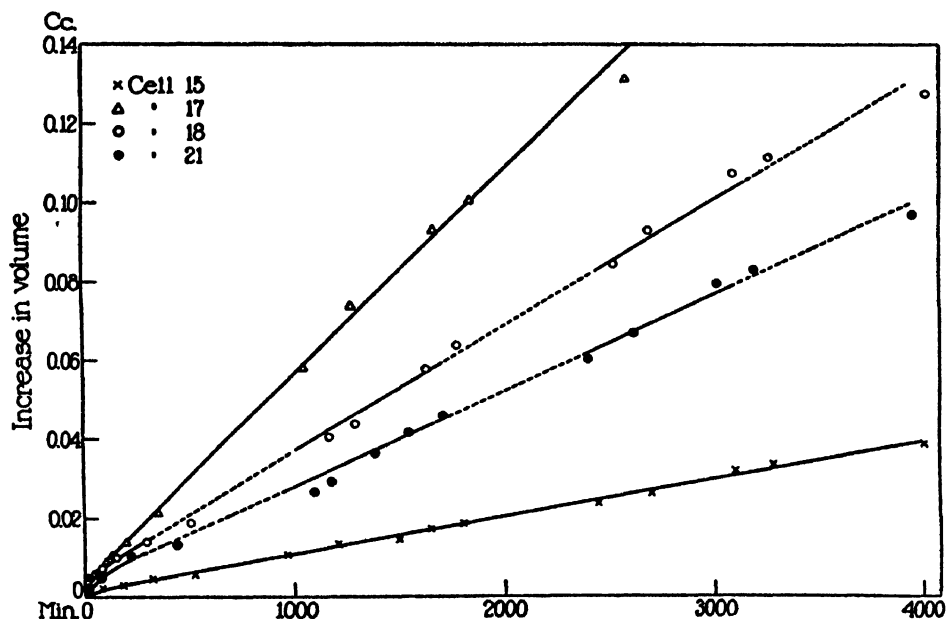


FIG. 2. Rate of increase of sap volume of impaled *Halicystis* cells in normal light (natural succession of daylight and darkness) from the moment of impalement. Note the initial rapid non-linear increase at the start. The curves for Cells 18 and 21 are drawn so as to show the falling off in rate during the hours of darkness and the gain in daylight. The broken portions represent 12 hours of darkness, the solid portions 12 hours of light. In drawing the curve these differences in rate have been disregarded so that the curve is approximately linear.

containing the cells in a large shallow tray through which passed a rapid current of sea water from the salt-water pumping system of the Biological Station.

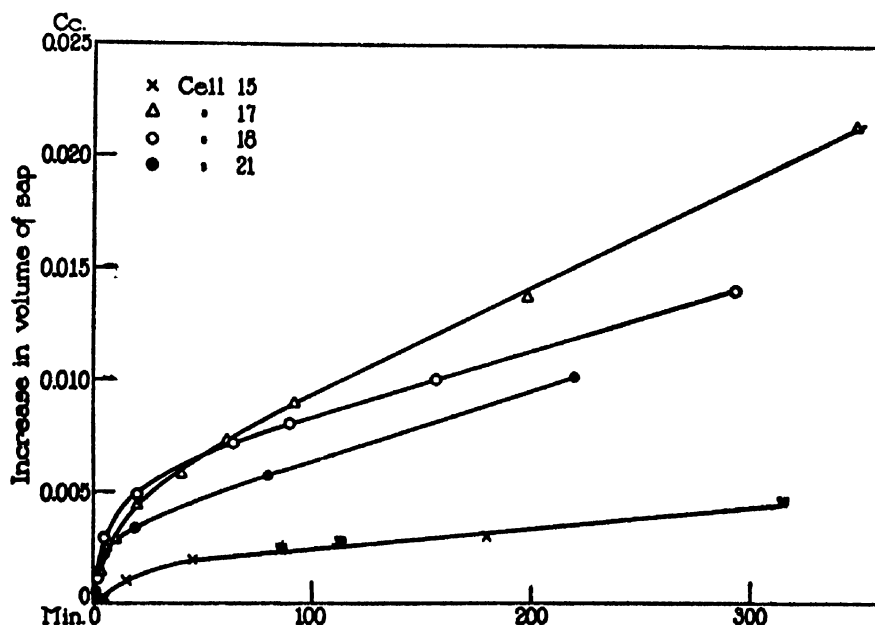


FIG. 3. A large scale representation of the early part of the curves of Fig. 2, illustrating the initial non-linear rate of volume increase.

TABLE I

Increase in Volume of Sap and in Moles of Halide in Impaled Cells of Halicystis

Cell No.	Days	Increase in volume of sap	Increase in volume of sap	Area of surface	Increase in moles halide per day	Increase in volume of sap per day
		cc.	per cent	cm. ²	per cent	per cent
1	5.0	0.095	33.0	2.108	6.6	6.6
2	5.0	0.101	31.0	2.328	6.2	6.2
3	4.6	0.269	45.7	3.394	9.4	9.9
4	3.9	0.141	17.5	4.189	4.6	4.5
Average					6.7	6.8

The increase in the moles of electrolyte in the sap (equal to molar concentration times volume increase in liters) was determined as the increase in the moles of halide. Since the cell sap of *Halicystis Osterhoutii* is chiefly a solution of sodium chloride, it was considered unnecessary to determine sodium and potassium as well in order to calculate moles of electrolyte.

The halide analyses were carried out electrometrically by titration with standard silver nitrate. The volume of each cell to be impaled was first determined by the method previously described and the number of moles of electrolyte originally present was calculated by using for the halide concentration in every case, the average halide concentration of a number of unimpaled cells from the same collection as the impaled cells. Since the natural variation in halide concentration is comparatively small this method gives sufficiently accurate results.

For the determination of the rate of increase in volume and in moles a group of unimpaled cells was kept under the same conditions as the impaled cells. The volume of these unimpaled control cells was determined in the manner previously described.²

A difficulty which arose constantly in these experiments was the decrease in the volume of sap within the cell vacuole at the moment of impaling. Blinks has already spoken of the difficulties associated with impalement of *Halicystis*, due to the tendency of the envelope to tear rather extensively. This means that in many cases some sap escapes round the capillary as the cell is pierced, hence it is hard to estimate the shrinkage of the vacuole. In a few cases cells were impaled without the loss except into the capillary tube and from measurements of this initial capillary rise in these cases it was possible to determine the shrinkage of the vacuole. It is variable, but appears to be between 15 and 25 per cent of the unimpaled volume.

In calculating the per cent increase in volume and in moles this has been taken into account by making a correction of the initial volume of 20 per cent. It is interesting that the difficulty just discussed is almost entirely absent in *Valonia*. In the first place, on impalement the vacuole shrinks only a very little, and, in the second, in nearly all cases of successful impalement the cell wall clings to the capillary so closely that there is no spurting.

In most cases spurting occurred with *Halicystis* cells, and before measurements of the increase in volume were started the cells were allowed to recover in normal sea water for 24 hours or more. The volume time curves for a number of these cells are given in Fig. 1. The curves are typical and the cases chosen were selected so as to avoid too many overlapping points.

In a number of cases we were successful in impaling cells without loss around the capillary. In some of these cases measurements were started at once, in order to determine the shape of the curves at the start. The results for some of these experiments are given in Fig. 2 and on a larger scale in Fig. 3 for the early part of the runs. One of these cells, No. 4, has been included in Fig. 1 for comparison.

Table I gives the data for the beginning and end of four experiments for which sap analyses are available. In 17 other cases not reported

² Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1931-32, 15, 537.

in detail the cells were transferred to other experiments and no sap analysis is available.

The control cells (unimpaired) gained 6.71 per cent in volume of sap in 8 days and 17.76 per cent in 18 days. The average daily increase was taken as 0.99 per cent.

DISCUSSION OF RESULTS

Figs. 1, 2, and 3 show that generally the behavior of the *Halicystis* cells was like that of the *Valonia* cells. Immediately after impalement there was a rapid increase in volume which was succeeded after a few hours by a slower increase, approximately linear with time, which lasted until the end of the experiment. The longest experiments described in this paper lasted 5 days, after which the cells were used in other experiments, and it seems not improbable that in the case of *Halicystis*, barring accidents, the linear increase in volume might go on indefinitely.³ In the case of *Valonia* the experiments were all terminated by the end of 48 hours because of the difficulty of keeping the capillary from plugging for long periods. But it seems probable that with *Valonia* also, as long as the capillary was kept free, the linear rate of increase of volume would continue.

In the case of *Halicystis*, although the general course of the curve after the first rapid uptake of water is linear, the curves are actually a series of waves. This is clearly brought out in Fig. 2 where a rather large scale is used. Some of the deviations from the linear curve are undoubtedly the result of experimental error but there is a certain regularity about them which we are able to identify definitely with the illumination. During the dark hours, the rate dropped off appreciably but it recovered promptly in daylight so that the net result was nil and the average curve is a straight line.

In order to bring out the periodic nature of the process under the influence of light two of the curves have been roughly divided into light and dark periods, the dotted portions representing 12 hours of darkness, roughly from 7 P.M. to 7 A.M., and the solid portions each

³ As a matter of fact cells used in other experiments lasted up to 20 days on the capillary and it is our impression that but for the unavoidable disturbances incident to the measurement of the capillary rise they might have lasted much longer.

representing 12 hours of light. The decrease in darkness and the increased rate in the light is fairly evident. These findings are in agreement with other light and dark experiments to be described in another paper.

Referring to Table I we find that the rate of increase of volume and of moles of halide (equivalent to moles of electrolyte) were parallel. This means that the halide concentration remained practically constant. The average percentage increase in volume per day in this group was 6.8 per cent, or if we apply a correction of 20 per cent to the initial volume of the cells to account for the shrinkage on impalement, the daily percentage increase in volume was 8.5 per cent.

This may be compared with the average daily increase of volume of 9.08 for 14 cells all drawn from an old collection, or on the corrected basis of 11.35 per cent. We have elected to compare these 14 cells which include the 4 of Table I because these were of the same collection as the unimpaled cells. The remaining 7 cells were of a much more recent collection and this apparently has some effect on the rate, since the corrected daily volume increase in this case was 19.9 per cent or rather less than twice as much as with the older cells. This difference may have some significance.

Neglecting these last younger cells for the reason that we have no figures for the rate in young unimpaled cells we see that volume increase and hence electrolyte increase was between 9 and 11 times as fast in the impaled cells as in the intact cells. This may be compared with the 15-fold increase observed when *Valonia* cells were impaled. We may say that the increase in rate produced by impalement is approximately the same in *Valonia* and in *Halicystis*.

In the case of *Valonia* we were not able to compare rates with the surface area, because the cells vary so much and are frequently so irregular in shape that we cannot relate the surface to the volume. In the case of *Halicystis* we can assume that the cells are spherical, particularly if they are small cells or have been kept a long time in the laboratory, under which condition they tend to round out to spheres. And if they are spheres we can calculate A , the surface area, from the volume.

From the calculations it appears that there is very little correlation between the surface area and the rate of uptake of water and electro-

lytes. However, when different cells are compared the average rate of increase of volume per day per square centimeter of surface for the first 14 cells was 0.013 cc. and for the other 7 cells 0.022 cc., or nearly twice as great. In the latter case, as pointed out already, this may be correlated with the time the cells remained in the laboratory.

In the case of the unimpaled cells the total surface area for 4 cells from the original volumes was 9.658 cm.² And ignoring the small increase in surface area during the volume increase this means that the average volume increase per day per square centimeter of surface was 0.015 cc., so that on this basis the rate is increased somewhat less than 9-fold due to impalement. In the calculation of the surface no correction was made for volume shrinkage on impalement. Such an allowance would raise the figure somewhat, possibly to nearly 10.5-fold, but we need not, in view of other uncertainties, make the calculation here. On either volume or surface basis therefore we can conclude that the rate of uptake of electrolyte and of water increases about 10-fold when the restriction due to the cellulose wall is removed by impalement.

As in the case of *Valonia* the osmotic concentration⁴ of the sap in the intact cell is slightly greater than that of the sea water. The freezing point of the sea water passing through the Biological Station pumping system varies slightly from time to time, but in comparison the freezing point of the sap of *Halicystis* kept in a flow of this sea water always has a slightly greater freezing point depression. For example, in one case the freezing point of the sea water was -2.030°C . and the freezing point of the sap of cells kept in it was -2.059°C . The usual difference of freezing point between sea water and the sap of *Valonia* is considerably greater than this. For example, in one case when the freezing point of the sea water in contact with the cells was -2.021°C . the freezing point of the *Valonia* sap was -2.150°C . Qualitatively this difference can be observed in the turgidity of the cells. *Halicystis* cells are invariably soft and rubbery to the touch, *Valonia* cells are firm and hard. In the case of *Valonia* we were unable to determine by calculation if the early more rapid entrance of

⁴ The osmotic concentration is unity when the freezing point depression equals 1.864; i.e., the freezing point depression of 1 mole of non-electrolyte dissolved in 1 liter of aqueous solution.

water immediately after impalement represents the more rapid entrance of water than electrolyte with a corresponding adjustment of the osmotic concentration of the sap to that of the sea water. But it seems probable that this is the case.

In the case of *Halicystis* the difference in osmotic concentration between sap and sea water is much less. The osmotic concentration of sap is $2.059 \div 1.864 = 1.1046$ M. Now in the case of cell 17 (Fig. 3) the initial period of rapid rise lasted about 92 minutes during which time the volume increased by 0.0088 cc., but we may suppose that only part of this was pure water, since electrolytes could enter also and it is a reasonable assumption that the rate of electrolyte entrance might be constant throughout the experiment. Therefore part of the increase in volume may be considered as the addition of undiluted sap to the volume. Interpolating from the straight part of the curve which apparently represents the increase in volume without dilution we find that in 92 minutes the amount of undiluted sap entering would be approximately 0.0045 cc. The original volume of the cell was 0.348 cc. Hence the calculated osmotic concentration should be approximately 1.0913 M. But the osmotic concentration of the sea water in contact with the cell was approximately $2.030 \div 1.864 = 1.0891$ M, a negligible difference.

On the other hand, similar calculations in the case of cell 15 gave for the probable osmotic concentration of the sap at the end of the rapid initial volume increase 1.0986 which suggests that the complete osmotic adjustment did not occur in this case. The evidence in this connection from halide analysis is also not decisive because the calculated change of halide concentration corresponding to complete osmotic adjustment is smaller than the natural variations in halide concentration among normal cells.

On the whole, therefore, the evidence while pointing to some osmotic adjustment does not support decisively the view that complete osmotic equality is attained. From a theoretical viewpoint, however, we think it desirable to assume that osmotic equality does occur on impalement.

We shall assume at first, that, just as in the case of *Valonia*, the entrance of sodium and potassium occurs by the diffusion of NaX and KX in the aqueous layer of the protoplasm, where X is the anion

of a weak acid HX elaborated by the protoplasm. In the present case, since the concentration of potassium in the sap is very small, we shall discuss sodium entrance only.⁵ In a previous paper we have suggested that the flux of M (M may be sodium or potassium) across a unit area of protoplasm of unit thickness is given by the equation

$$\frac{dM}{dt} = D^{MX} \{ [MX]_{epo} - [MX]_{epi} \}$$

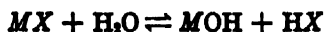
and this with suitable simplifications reduces to

$$\frac{dM}{dt} = k_{\text{coll.}} D^{MX} \{ [M]_o (\text{OH})_o - [M]_i (\text{OH})_i \}$$

where

$$k_{\text{coll.}} = \frac{f_o^{\text{Na}} f_{epo}^{\text{HX}} S_{eop}^{\text{NaX}} [\text{HX}]_{epo}}{f_{eop}^{\text{NaX}} S_{eop}^{\text{HX}} k_{\text{hydrolysis}}^{\text{NaX}}}$$

In these equations D is the diffusion constant, S is the partition coefficient, f is the activity coefficient, and $k_{\text{hydrolysis}}^{\text{NaX}}$ is the thermodynamic hydrolysis constant of the reaction



Round brackets indicate activities and square brackets concentrations. The subscripts o and i refer to the bulk of the sap and sea water respectively, and the subscripts epo and eop refer to a pair of adjacent unstirred layers in the protoplasm and sea water respectively in which the solute and solvents are in equilibrium across the interface: epi and eip refer to similar layers at the sap-protoplasm interface.

In the derivation of this equation it was assumed that corresponding activity coefficients and partition coefficients in the sap and sea water are equal. HX is also considered to be distributed equally throughout the protoplasm.

The rate of entrance of water is given by the flux of water across the non-aqueous protoplasm, *viz.*

$$\frac{d\text{H}_2\text{O}}{dt} = D^{\text{H}_2\text{O}} \{ [\text{H}_2\text{O}]_{epo} - [\text{H}_2\text{O}]_{epi} \}$$

⁵ The situation is complicated by the fact that halide is entering the cell simultaneously. A mechanism whereby halide may enter will be discussed elsewhere.

Now the entrance of water and electrolyte goes on under steady state conditions. Hence the ratio of the rates of entrance of electrolyte to water remains constant while only the volume of the sap increases. There is good evidence that the rate of entrance of water takes place much more rapidly than the rate of electrolyte entrance. Thus, as we showed in a previous paper, even when the sea water is diluted extensively the adjustment of the osmotic pressure between the sea water and the sap of an impaled *Halicystis* cell takes place almost entirely by entrance of water into the sap and hardly at all by loss of electrolyte.⁶

If this is so then in the impaled cell we should expect the osmotic concentration of the sap at the steady state to be very close to that of the sea water, for when the cell takes in electrolyte and thereby raises the osmotic concentration by a small amount water can enter at once, since there is no volume restriction due to the cell wall. This would tend to abolish the osmotic gradient.

When the cell is intact we expect a different steady state concentration in the sap, for then water can enter only when the cell wall grows and so is able to enclose more space. Hence the rate of water entrance is decreased, and in order to decrease the rate of electrolyte entrance $\{[Na]_i(OH)_o - [Na]_i(OH)_i\}$ must decrease. This can only happen by $[Na]_i$ increasing since the other concentrations are fairly well fixed. We may note that the situation is somewhat different from the situation in Osterhout's guaiacol model, where the rate of uptake of water is increased as the concentration of electrolyte in the inner phase increases. In the present case the rate of uptake of water cannot increase as the concentration of the electrolyte in the sap increases, because of the cell wall restriction. Consequently all the adjustment to the new steady state depends on $[Na]_i$ increasing. We need not predict at what point the steady state will be established but it is clear that it must be much higher than 0.6 M for $(OH)_o$ is at least 100 times $(OH)_i$ hence $[Na]_i$ may increase many fold without decreasing the gradient appreciably.⁷

⁶ Jacques, A. G., *J. Gen. Physiol.*, 1938-39, **22**, 743.

⁷ Let us consider a cell containing 1 ml. of sap. In the impaled cell the cell sap gains in about 10 days (10 per cent increase in volume per day, the concentration of electrolyte being 0.6 M) 1 ml. of water and 0.6 millimole of electrolyte. Now suppose an impaled cell could be suddenly made intact. The cell would

It was because practically the same steady state was obtained in both intact and impaled *Valonia* cells, even though there was a 15-fold change in the rate of entrance of water and electrolyte, that we were led to suggest a regulatory mechanism which operates by changing the partition and diffusion coefficients of MX in the non-aqueous protoplasm. It was suggested that the mechanism operates about as follows. In the intact cell a little electrolyte enters thereby raising the osmotic concentration of the sap. As a result water enters and stretches the cellulose wall to the limit. Electrolyte continues to enter but water cannot until more cellulose is produced. Then as the osmotic pressure of the sap increases, water is withdrawn from the protoplasm. This does not require any change in the volume (sap + protoplasm) inside the cellulose envelope, but it does change the properties of the non-aqueous protoplasm, by lowering the partition coefficient of MX , so that $\{MX_{sap} - MX_{epi}\}$ is decreased.⁸ At the same time the viscosity of the protoplasm is increased and D^{MX} is decreased.

On the whole we think that the same explanation may apply in the present case. Before discussing it let us consider an alternative mechanism by which sodium is assumed to enter the cell as sodium chloride.

According to the analysis of Bermuda sea water and *Halicystis* sap⁹ $[Na]_o = 0.498$ M, $[Cl]_o = 0.580$ M, $[Na]_i = 0.557$ M, and $[Cl]_i = 0.603$ M. Since the ionic strengths of the sap and sea water are quite close to each other the activity coefficients may be taken as equal.¹⁰

now gain 1 ml. of water in about 100 days under these conditions, and if the rate of entrance of electrolyte remained unchanged it would gain 6 millimoles of electrolyte in the same time and the concentration would become $6.6 + 2 = 3.3$ M. But if the concentration were 3.3 M the gradient would be reduced from approximately 5.94×10^{-7} to 5.67×10^{-7} . This very approximate calculation is made as follows, $[Na]_o = 0.6$ M, $[OH]_o = 10^{-8}$ M, $[Na]_i = 0.6$ M, $[OH]_i = 10^{-8}$ M, $[Na]_o \times [OH]_o = 6 \times 10^{-7}$, and $[Na]_i \times [OH]_i = 0.06 \times 10^{-7}$, whence the difference is 5.94×10^{-7} . When $[Na]_i$ is taken as 3.3 M, $[Na]_i \times [OH]_i = 0.33 \times 10^{-7}$ and the difference becomes 5.67×10^{-7} . That is, it would decrease less than 10 per cent. We need not attempt to fix the steady state concentration more closely.

⁸ Regarding *epo*, *cop*, *epi*, and *eip* see p. 305.

⁹ Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, 5, 225. Blinks, L. R., and Jacques, A. G., *J. Gen. Physiol.*, 1929-30, 13, 733.

¹⁰ The ionic strength of Bermuda sea water, according to the calculation of

Hence the chemical potential of sodium chloride is greater in the sap than in the sea water. Consequently NaCl as such cannot diffuse from the sea water to the sap without the cell supplying energy. If sodium chloride diffuses as such from the sea water to the sap we may write for the flux of NaCl

$$\frac{d[\text{NaCl}]}{dt} = \frac{D^{\text{NaCl}}}{h} \{[\text{NaCl}]_{\text{spo}} - [\text{NaCl}]_{\text{spi}}\} \quad (1)$$

or

$$= \frac{D^{\text{NaCl}}}{h} \{S_{\text{sap}}[\text{Na}]_o[\text{Cl}]_o - S_{\text{spi}}[\text{Na}]_i[\text{Cl}]_i\}^* \quad (2)$$

* The assumption and steps by which we arrive at equations similar to the present one have been applied in several forms in another paper (Jacques, A. G., *J. Gen. Physiol.*, 1938-39, 22, 147) and they need not be considered in detail here.

In order for the right hand term of the gradient to become less than the left hand in *Halicystis* S_{spi} must be less than S_{sap} . But in any diffusion system to which no energy is supplied S_{sap} and S_{spi} must obviously come closer together as $[\text{Na}]_o[\text{Cl}]_o$ and $[\text{Na}]_i[\text{Cl}]_i$ come closer together until when these products are equal the partition coefficients are also equal. In a system such as *Halicystis* it seems not impossible that the required energy may be available.

Suppose the energy of the cell were directed to the production of a substance which depresses the solubility of sodium chloride in the non-aqueous phases. This would lower the partition coefficients. But in order for such a substance to lower S_{spi} differentially it would be necessary for it to be present in higher concentrations at the sap-protoplasm interface than at the sea water-protoplasm interface. This seems possible, for example, if the substance is produced only at the sap-protoplasm interface and has a low rate of diffusion in the non-aqueous protoplasm. Thus we can imagine a "steady state"

Zscheile (Zscheile, F. P., Jr., *Protoplasma*, 1930, 11, 481) is 0.7212, and the ionic strength of *Halicystis* sap by our calculation is 0.6326. By reference to the data of Harned (Harned, H. S., in Taylor, H. S., *A treatise on physical chemistry*, New York, D. Van Nostrand Company, Inc., 2nd edition, 1931, 1, 772) we see that between these two ionic strengths the activity coefficient of sodium chloride changes hardly at all.

in which the "depressant" agent is being produced rapidly at the sap-protoplasm interface and is diffusing slowly to the sea water-protoplasm interface, so that a rather steep but constant gradient of the substance is set up across the protoplasm. S_{si} then remains continuously less than S_{so} and the flux of NaCl is inward in spite of the fact that $[Na]_i [Cl]_i > [Na]_o [Cl]_o$.

The part played by the aqueous phases may be important. Since the depressant agent is able to displace the polar sodium chloride from the non-polar protoplasm, it must itself be relatively more non-polar. Hence its partition coefficient must be high. At the sap-protoplasm interface we have the relationship

$$S_{si}^a [a]_{si} = [a]_{si}$$

where a is the depressant agent. In the steady state it seems likely that $[a]_{si} = [a]_i$ (where $[a]_i$ is the concentration in the body of the sap) because the volume of the sap is limited.

At the sea water-protoplasm interface, however, $[a]_{so} > [a]_o$ since the sea water is not limited in volume. Hence there is a loss of the agent to the sea water, and in the steady state this may take place at such a rate that the decrease in the *epo* layer is just made up by the diffusion of the agent from the sap-protoplasm interface. This steady loss is important since if the depressant were produced continuously and did not escape from the protoplasm no steady state would be possible. Instead the concentration of the depressant would eventually become equal at the two interfaces when, of course, its differential effect on the partition coefficients of sodium chloride at sea water and sap interfaces would disappear.

Osterhout¹¹ has suggested that the protoplasm of such large plant cells as *Halicystis* consists of two non-aqueous layers, X and Y , adjacent to the sea water and sap respectively, and W , an aqueous layer, between them. It might be supposed that if the X and Y layers have different properties S_{so} and S_{si} must necessarily be different, and that accumulation of sodium chloride as NaCl could occur on this account alone. It is easy to see that this could not be so thermodynamically. As a matter of fact the system does not

¹¹ Osterhout, W. J. V., *Ergebn. Physiol.*, 1933, **35**, 1014.

differ fundamentally from a system with only one non-aqueous layer separating sea water and the sap.

Suppose we have a three layer system in which the diffusion coefficients are different and suppose the thicknesses of the layers are h_s , h_w , and h_y . The total thickness of the system is small so that "steady state diffusion" prevails. The flux in all parts of the system is therefore the same. Jacobs¹² has shown for a two layer system of this type that the rate of transfer of solute is proportional to the gradient across the whole system. The separate gradients across each layer need not be considered. Extending Jacobs' reasoning to our three layer system we have for the flux across a unit area

$$\frac{d[\text{NaCl}]}{dt} = \frac{D_s D_w D_y}{D_y D_w h_s + D_y D_s h_w + D_s D_w h_y} \{ [\text{NaCl}]_{spi} - [\text{NaCl}]_{spo} \} \quad (3)$$

which is very similar to the equation (1) set up for the flux in a single layer.

It might be suggested that the supposed diffusion of the depressant from the sap to the sea water resembles the movement of an ionogenic "diffusing agent" which Teorell¹³ has shown is capable of bringing about accumulation in non-living systems. The resemblance is superficial since the depressant is not supposed to be ionogenic and hence cannot furnish the ions of different mobility required to produce the electrical stress which according to Teorell's theory is the force which brings about the redistribution of other ions in the system.

In an average *Halicystis* cell $[\text{Na}]_o [\text{Cl}]_o = 0.289$ and $[\text{Na}]_i [\text{Cl}]_i = 0.336$. Hence if the ratio $S_{si} \div S_{so}$ could be maintained at a value slightly greater than 1.16 sodium could enter the cell as sodium chloride. Let us suppose for the moment that this condition is met in the intact cell. When the cell is impaled the rate increases about 10-fold hence if D^{NaCl} remains unchanged $S_{so} [\text{Na}]_o [\text{Cl}]_o - S_{si} [\text{Na}]_i [\text{Cl}]_i$ must increase 10-fold. Since there is only a very slight decrease of $[\text{Na}]_i [\text{Cl}]_i$ in the impaled cell the change must be effected through the partition coefficients. However, it is difficult to see

¹² Jacobs, M. H., *Ergebn. Biol.*, 1935, 12, 70.

¹³ Teorell, T., *Proc. Nat. Acad. Sc.*, 1935, 21, 152; *J. Gen. Physiol.*, 1937-38, 21, 107.

how the results we observe could come about through the depressant a . There is no reason to suppose that its rate of production would be increased by impalement. But since the volume of the sap increases faster in the impaled cell, the loss of a to the sap will be greater, hence $[a]_{epi}$ will be smaller.

This effect may be small and the increase in S_{stp} due to the decrease in the concentration of the agent may in part be compensated by the smaller concentration of a in the epo layer which will cause S_{sep} to increase. The net effect on the partition coefficient ratio will probably be nearly zero. And in any case there seems to be no possibility that it can be so increased by the depressant that the gradient term increases 10-fold.

Thus even if sodium diffuses in by the mechanism suggested above it seems necessary to assume that there is an additional mechanism whereby the gradient is altered when the cell is impaled.

As pointed out above we assume that the additional mechanism operates by withdrawing water from the non-aqueous protoplasm to reduce both S_{sep} and S_{stp} and at the same time by increasing the viscosity of the non-aqueous phase to reduce the diffusion coefficients. According to this view in the intact cell some of the energy of the cell is directed towards decreasing the rate of uptake of electrolyte. When the cell is impaled the energy is no longer expended in this way and without the need for an increase in the energy output of the plant the rate of uptake of electrolyte can increase.¹⁴

However, recent results of Blinks, Darsie, and Skow¹⁵ on the Pacific Coast *Halicystis ovalis* indicate that on impalement the cell consumes oxygen at a more rapid rate than normal and this keeps up for some time "even for several days." That is to say on impalement the en-

¹⁴ An alternative mechanism for keeping the osmotic concentration of the cell sap from increasing much above that of the sea water in the intact cell has been suggested to us by Dr. T. Shedlovsky. He suggests that when the cell is stretched to the limit the protoplasm ruptures momentarily and as a result sap escapes and the vacuole volume decreases. Then the rupture heals and as the cellulose wall is not now stretched to the limit, water can enter to stretch it, thereby reducing the osmotic concentration. This process is supposed to be repeated an indefinite number of times.

¹⁵ Blinks, L. R., Darsie, M. L., Jr., and Skow, R. K., *J. Gen. Physiol.*, 1938-39, 22, 255.

ergy output of the cell is increased. Hence in contrast to the view expressed above it might be suggested that the increased rate of uptake of electrolyte is in some way associated with this. However, although in the present paper, the longest time during which the rate was followed was 5 days, in a forthcoming paper¹⁶ we shall describe experiments in which the accelerated rate of uptake over that of the intact cell continued for periods up to 15 days and it scarcely seems likely that the accelerated rate of oxygen consumption would continue so long.

Possibly, however, the more rapid non-linear rate of increase in volume during the first few hours is associated with increased energy output. But it seems just as likely that it is associated with the partial dissipation of the excess osmotic energy of the sap over that of the sea water.

Since in the case of the impaled *Valonia* cell the experiments were terminated in 48 hours or less, two questions arise. First, does impalement result in increased energy output for several days in *Valonia* as it does in *Halicystis ovalis*, and second, is the increased rate of uptake of electrolyte associated in any way with increased energy output? We think that the last question should be answered negatively, for the reason that *Valonia* and *Halicystis Osterhoutii* on impalement behave qualitatively and quantitatively nearly alike, and, as pointed out already, in the latter case it seems unlikely that the increased energy output would persist as long as the increased rate of uptake lasted.

In the case of *Valonia*, measurements on the sap made in two cases after 48 hours of increased electrolyte uptake due to impalement, failed to show any definite change in the pH. Yet if the cell were to continue to respire more rapidly on impalement we might possibly expect to find the pH of the sap lower than normal.

Between the two alternatives entrance as NaX or as NaCl by the aid of a depressant, there is little to choose as far as the evidence goes, but in the case of certain plants such as *Nitella*, where the osmotic pressure and the concentrations of most of the ions in the sap are greater than in the surrounding medium, it seems possible that favor-

¹⁶ Jacques, A. G., *J. Gen. Physiol.*, 1939-40, 23, in press.

able gradients may depend on substances present in larger concentration at the sap-protoplasm interface, which depress the solubility of the salts in the non-aqueous phases.

SUMMARY

When cells of *Halicystis* are impaled on a capillary so that space is provided into which the sap can migrate, the rate of entrance of water and of electrolyte is increased about 10-fold. In impaled *Valonia* cells the rate is increased about 15-fold.

After a relatively rapid non-linear rate of increase of sap volume immediately after impalement (which may possibly represent the partial dissipation of the difference of the osmotic energy between intact and impaled cells) the volume increases at a linear rate, apparently indefinitely.

Since the halide concentration of the sap at the end of the experiment is (within the limits of natural variation) the same as in the intact cell, we conclude that electrolyte also enters the sap about 10 times as fast as in the intact cell.

As in the case of *Valonia* we conclude that there is a mechanism whereby in the intact cell the osmotic concentration of the sap is prevented from greatly exceeding that of the sea water. This may be associated with the state of hydration of the non-aqueous protoplasmic surfaces.

BIOELECTRIC POTENTIALS IN VALONIA

II. EFFECTS OF ARTIFICIAL SEA WATERS CONTAINING LiCl, CsCl, RbCl, OR NH₄Cl

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Measurements of P.D. across the protoplasm of cells of *Valonia macrophysa*, Kütz., have furnished valuable information¹ as to the behavior of the protoplasm toward Na⁺, K⁺, and Cl⁻ ions. It has accordingly seemed desirable to extend this study to ions of similar chemical properties, not present in significant amounts in ordinary sea water. The present report gives the results of a few exploratory measurements² using various dilutions of modified sea waters in which LiCl, CsCl, RbCl, or NH₄Cl was substituted for NaCl and KCl.

In the experiments with various dilutions of natural and KCl-rich sea waters,¹ P.D.-time curves of two different types have been observed.

When natural sea water is replaced by undiluted KCl-rich sea water, the P.D. rises³ rapidly to a maximum, falls to a minimum, and then rises more slowly to a second maximum. This characteristic fluctuation of the P.D. (found also with *Valonia* sap and with pure 0.6 molar KCl) has been attributed⁴ to changes caused

¹ (a) Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, **13**, 445. (b) Damon, E. B., *J. Gen. Physiol.*, 1932-33, **16**, 375. (c) Damon, E. B., *J. Gen. Physiol.*, 1937-38, **21**, 383.

² The experiments reported here were performed at Bermuda in 1930. It was originally planned to make a detailed study of the effects of each of these salts, but this program has actually been carried out only in the case of KCl.^{1b, c} Since this work has been interrupted and cannot be continued by the writer, it seems worth while to publish these results in spite of their fragmentary nature.

³ In the experiments reported in this paper, the P.D. across the protoplasm is in all cases directed inward, in the sense that positive current tends to flow from the external solution through the protoplasm into the vacuolar sap.

⁴ (a) Damon, E. B., *J. Gen. Physiol.*, 1929-30, **13**, 207. (b) Damon, E. B., *J. Gen. Physiol.*, 1931-32, **15**, 525.

by the penetration of K^+ into the aqueous middle layer⁵ of the protoplasm. Although the later changes in P.D. are complex, it is found that the initial rise in P.D. with KCl-rich sea waters is reproducible, and varies in a regular manner with the concentration of potassium, C_K , in the external solution according to the equation

$$P.D. = 59.1 \log (BC_K + D) \quad (\text{mv. at } 25^\circ \text{ C.})$$

where B and D are constants. It is assumed that the first maximum in the P.D.-time curve occurs before the concentrations in the inner layers of the protoplasm have been affected by the penetration of K^+ ; in other words, that only the external surface layer of the protoplasm (the X layer) is concerned in the initial rise in P.D.

Similar P.D.-time curves are observed with small dilutions of KCl-rich sea waters, the initial rise in P.D. decreasing as the dilution is increased. With these solutions, also, K^+ plays an important part in determining the P.D.

With diluted natural sea water and with high dilutions of KCl-rich sea waters, however, the P.D.-time curves have a different shape. When these solutions are substituted for ordinary sea water, the P.D. rises rapidly to a definite value at which it remains approximately constant for some time; the P.D. increases with increasing dilution. The P.D. is independent of small changes in the concentration of K^+ in these solutions. The greater is the concentration of K^+ in the undiluted sea water, the higher is the critical dilution at which K^+ ceases to influence the P.D. In sea waters containing both KCl and NaCl, the concentration effect above the critical dilution is determined solely by the activity of NaCl in the external solution.

From these results, certain inferences have been drawn in regard to the diffusion of K^+ , Na^+ , and Cl^- in the outer surface layer of *Valonia* protoplasm (the X layer).

The concentration effect with these sea waters above the critical dilution has been interpreted as a diffusion potential in the X layer of the protoplasm, involving only the Na^+ and Cl^- ions which are coming out from the vacuole. (The outward diffusion of K^+ from the vacuole is evidently prevented by the mechanism responsible for the accumulation of KCl in the cell sap.) The relative mobilities of Na^+ and Cl^- in the X layer may then be calculated from this diffusion potential with the help of the familiar Nernst equation. If the mobility of the Cl^- ion is arbitrarily taken as unity, the mobility of Na^+ is found¹⁶ to be 0.11.

The electrical potential gradient in the X layer produced by the outward diffusion of Na^+ and Cl^- tends to oppose the inward diffusion of other cations from the external solution. This explains the failure of K^+ to enter the protoplasm and influence the P.D. when the dilution is greater than the critical value. While it has proved possible to calculate the critical dilutions for KCl-rich sea waters, this calculation is based on certain assumptions which cannot be applied without

⁵ For the theory of protoplasmic layers, see Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, 11, 83; *Biol. Rev.*, 1931, 6, 369; *Ergebn. Physiol.*, 1933, 35, 1013.

modification to other cations than K^+ (or to other organisms than *Valonia*). Nevertheless, similar phenomena are to be expected with *Valonia* when other cations are substituted for Na^+ and K^+ in diluted sea water.

The apparent relative mobility of K^+ in the outer or *X* layer of the protoplasm has been calculated from the initial rise in P.D. in undiluted KCl-rich sea waters, assuming that this change in P.D. arises from a new diffusion potential in the *X* layer. If the mobility of Cl^- is taken as unity, the apparent mobility of K^+ (recalculated to agree with the new value of 0.11 for Na^+) is found to be 18. In this calculation, the additional assumption was made that the partition coefficients of KCl and NaCl between water and the *X* layer are equal. Since it is not improbable that the partition coefficient for KCl is actually considerably higher than that for NaCl, the value, 18, may be too high. The sign and magnitude of the concentration effect with small dilutions of KCl-rich sea waters, however, indicate that the mobility of K^+ must be several times greater than that of Cl^- .

We may now consider some similar experiments using sea waters containing Li^+ , Cs^+ , Rb^+ , or NH_4^+ .

The composition⁶ of the modified sea waters used in these experiments is given in the following table, where the symbol, M, is used to represent Li, or Cs, or Rb or NH_4 .

M	0.500 molar	Cl	0.570 molar
Ca	0.011 "	Br	0.001 "
Mg	0.054 "	SO_4	0.028 "
		HCO_3	0.003 "

The pH of each solution was adjusted to the same value as that of ordinary sea water as shown by the color of thymol blue. These stock solutions are designated as *LiCl-sea water*, etc. In some experiments these stock solutions were diluted with natural sea water; the resulting solutions are described by such expressions as *0.1 molar RbCl in sea water*. For studying the concentration effect, the stock solutions were diluted with a solution of glycerol, 8.7 per cent by weight, in distilled water. (This solution is approximately isotonic with Bermuda sea water.) Such a diluted solution may be called, for example, a *d-fold dilution of NH_4Cl -sea water*. Here, the dilution, *d*, represents the number of liters of diluted solution containing 1 liter of the stock solution.

⁶ Based on a formula for artificial sea water recommended by McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. No. 251*, 1917.

The apparatus and experimental methods have been described in earlier publications.⁴ To avoid waste in experiments with solutions containing RbCl and CsCl, the same technic was employed as in the measurements with natural *Valonia* sap.^{4b}

I

Modified Sea Water Containing LiCl

In its influence on the P.D. with *Valonia*, the Li^+ ion appears hardly distinguishable from the Na^+ ion, at least so far as the outer surface layer of the protoplasm (the X layer⁶) is concerned. In eleven meas-

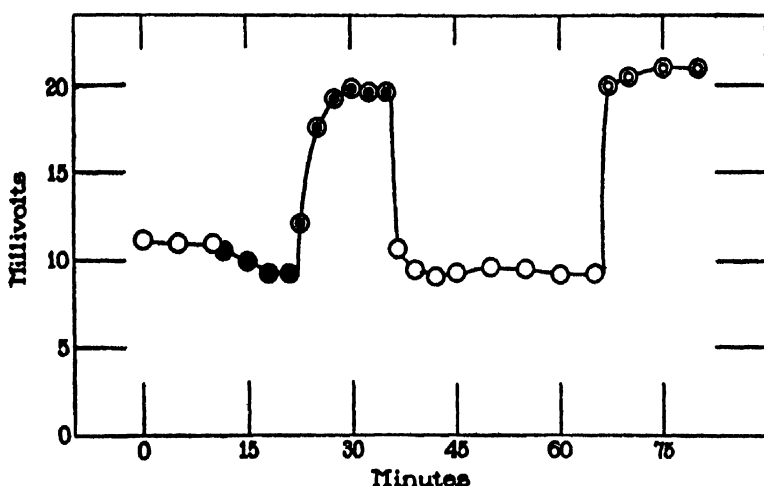


FIG. 1. P.D.-time curve showing the change in P.D. across *Valonia* protoplasm when ordinary sea water (open circles) is replaced by LiCl-sea water (shaded circles) and comparing the concentration effects with a twofold dilution of LiCl-sea water (double circles, shaded centers) and with an equal dilution of natural sea water (double circles, open-centers). LiCl-sea water contains 0.5 M LiCl.

urements with LiCl-sea water, the P.D.-time curves resembled the curves with potassium-free NaCl-sea water: the P.D. generally fell to a value less than the value in ordinary sea water, but greater than zero. Such a curve is shown in Fig. 1. A representative P.D.-time curve with potassium-free NaCl-sea water has been presented in an earlier paper.⁷

With both the NaCl-sea water and the LiCl-sea water, however, the behavior is liable to be erratic; these potassium-free solutions are

⁷ Damon, E. B., *J. Gen. Physiol.*, 1932-33, 16, 378 (curve marked $C_K = 0$).

evidently somewhat injurious and prone to cause secondary changes in the protoplasm. A LiCl-sea water containing the same concentration of K^+ as ordinary sea water would probably give better results. More prolonged measurements, which should be possible with such solutions, might reveal effects at inner layers of the protoplasm where Li^+ may behave differently from Na^+ .

With diluted LiCl-sea water, the P.D.-time curves are similar to the curves with diluted natural sea water; the concentration effect with LiCl-sea water, however, is somewhat smaller. Fig. 1 compares the concentration effects with twofold dilutions of LiCl-sea water and of natural sea water. Four such measurements gave values for the concentration effect with a twofold dilution ranging from 6 to 10 mv., average, 8 mv. One measurement with a fivefold dilution gave 22 mv. The values for two- and fivefold dilutions of natural sea water, reported in an earlier paper,^{1a} were 11.4 and 25.2 mv. From comparison of these concentration effects, we may conclude that the mobility of Li^+ in the *X* layer of *Valonia* is perhaps slightly greater than that of Na^+ .

II

Modified Sea Water Containing CsCl

The Cs^+ ion, like the Li^+ ion, resembles the Na^+ ion in its effect on the P.D. with *Valonia*. Two measurements with CsCl-sea water gave P.D.-time curves like the curves with potassium-free NaCl-sea water. One of these curves is shown in Fig. 2.

Three measurements of concentration effect with a fivefold dilution of CsCl-sea water showed P.D.'s somewhat higher than the P.D. with an equal dilution of ordinary sea water; this is illustrated in Fig. 2. The same result was obtained whether the diluted CsCl-sea water was applied after the diluted natural sea water, or before it as shown in the figure. This larger concentration effect might seem to indicate that the mobility of the Cs^+ ion in the *X* layer is less than that of the Na^+ ion.

It is not certain, however, that with the fivefold dilution the Cs^+ ion really plays any significant part in the P.D. It may be that this dilution is greater than the critical dilution for CsCl-sea water, and that only Na^+ and Cl^- ions diffusing out from the vacuole are actually

concerned in the P.D. The P.D. to be expected in such a case may be estimated by extrapolating the straight line which represents the P.D.-dilution curve of KCl-sea water⁸ above its critical dilution. The extrapolated value for a fivefold dilution is in good agreement with that observed with the diluted CsCl-sea water.

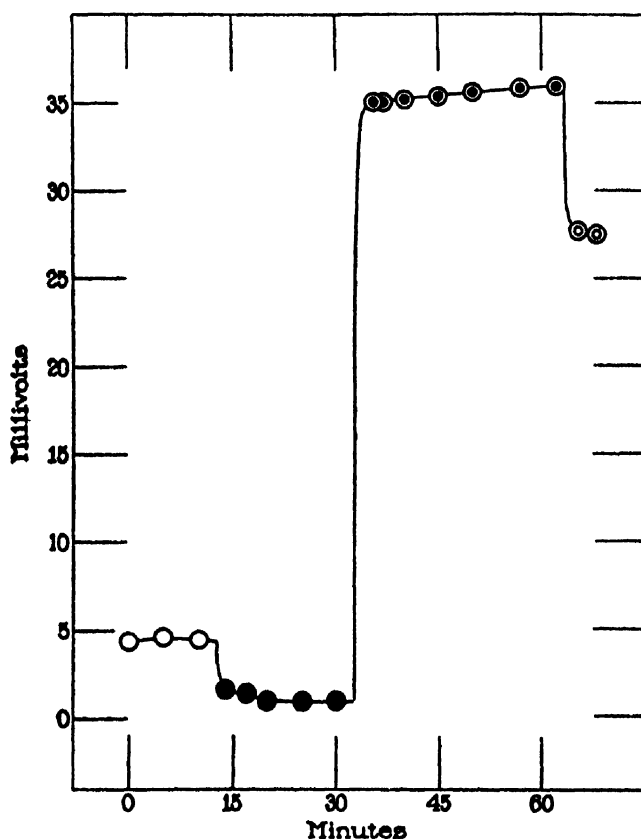


FIG. 2. P.D.-time curve showing the change in P.D. across *Valonia* protoplasm when ordinary sea water (open circles) is replaced by CsCl-sea water (shaded circles) and comparing the concentration effects with a fivefold dilution of CsCl-sea water (double circles, shaded centers) and with an equal dilution of natural sea water (double circles, open centers). CsCl-sea water contains 0.5 M CsCl.

Accordingly, while we may conclude from the data with undiluted CsCl-sea water that the mobility of Cs^+ in the X layer is not very different from that of Na^+ , these measurements of concentration effect do not show conclusively which of the two mobilities is the higher.

⁸ Damon, E. B., *J. Gen. Physiol.*, 1937-38, 21, 388, Fig. 2, curve A.

III

Modified Sea Waters Containing RbCl

Sea waters containing RbCl, like KCl-rich sea waters, increase the inwardly directed P.D. across the protoplasm of *Valonia*. The curves showing the variation of P.D. with time are more or less similar to the curves with KCl-rich solutions. With solutions containing RbCl, however, the second rise in P.D. (ascribed to changes at inner layers of the protoplasm) follows so soon after the initial change that the time curve shows only a point of inflection instead of a well defined maximum and minimum. P.D.-time curves of this type are found occasionally with KCl-rich solutions;⁹ in such cases, the value of the P.D. at the point of inflection is about the same as the value at the first maximum in curves of the usual KCl type.

Curve A of Fig. 3 shows a P.D.-time curve of this sort observed with RbCl-sea water. The initial change in P.D. (to the point of inflection) was about 53 mv. In another measurement with RbCl-sea water, the time curve had a well defined maximum and minimum, but here the initial rise was only 33 mv.

Three measurements with 0.1 molar RbCl in sea water gave P.D.-time curves in which the initial rise was indicated, at the best, by an ill-defined point of inflection. The best of these curves is shown as curve B in Fig. 3. In this curve, there is apparently an inflection point at about 21 mv. above the P.D. with ordinary sea water. In these experiments the P.D. finally reached surprisingly high values: in one case, the P.D. after 1 hour was 60 mv. greater than the value with ordinary sea water.

In two measurements with 0.05 molar RbCl in sea water the changes in P.D. were small and gradual. When these solutions were replaced by 0.1 molar RbCl in sea water, however, the P.D. rose rapidly and passed through a well defined maximum. This behavior will be discussed later.

The data furnished by the two measurements reported in curves A and B of Fig. 3 are probably the most reliable values which we have for calculating the apparent relative mobility of Rb^+ in the X layer of

⁹ Damon, E. B., *J. Gen. Physiol.*, 1929-30, 13, 215; 1932-33, 16, 384.

Valonia. This calculation is based on the same assumptions as were used in an earlier paper^{1b} in calculating the apparent relative mobility of K^+ . It is supposed that the initial change in P.D. (to the point of inflection) represents a diffusion potential set up in the *X* layer of the protoplasm. It is further assumed that the coefficients for the dis-

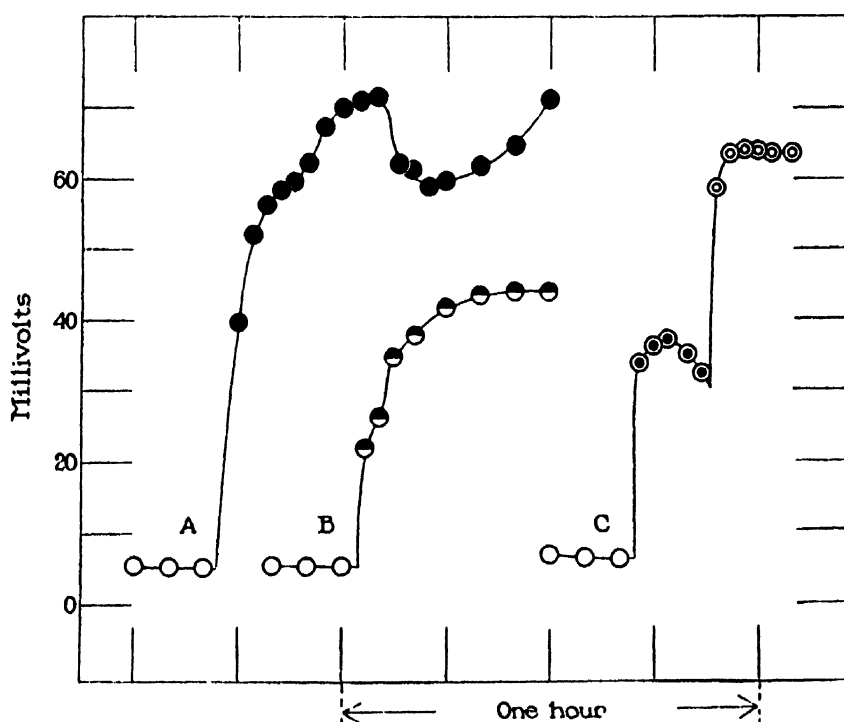


FIG. 3. Time curves showing changes in P.D. across *Valonia* protoplasm when ordinary sea water (open circles) is replaced by modified sea waters containing RbCl. In curve A the external solution is RbCl-sea water (shaded circles); in curve B it is 0.1 molar RbCl in sea water (half-shaded circles). Curve C illustrates the concentration effect with diluted RbCl-sea water: fivefold dilution, double circles, shaded centers; 25-fold dilution, double circles, open centers. RbCl-sea water contains 0.5 M RbCl.

tribution of NaCl, KCl, and RbCl between sea water and the *X* layer all have the same value. Using the values which have already been determined for the apparent relative mobilities of Na^+ , K^+ , and Cl^- , the relative mobility of Rb^+ may then be computed with the help of the equation (derived from the familiar Henderson formula) which was used in computing the relative mobility of K^+ . From the

measurement with RbCl-sea water (curve A) it is found that the mobility of Rb^+ is 11 times as great as that of Cl^- ; from the measurement with 0.1 molar RbCl in sea water (curve B), the value is found to be 10.

Since the mobility of Rb^+ in the X layer is much greater than that of Cl^- , and since the behavior of Rb^+ is similar to that of K^+ , it is to be expected that the concentration effect with RbCl-sea water will resemble the concentration effect with KCl-sea water. That is, we may expect that the initial change in P.D. produced by diluted RbCl-sea water (if the dilution is not too great) will be less than the corresponding change produced by the undiluted solution. We may expect further that as the dilution is increased a critical value will be reached, above which Rb^+ plays no part in the P.D., and the P.D. increases with increasing dilution. The P.D.-dilution curve will therefore pass through a minimum.

These predictions are confirmed by measurements with fivefold and 25-fold dilutions of RbCl-sea water. Thus, three measurements with the fivefold dilution showed an initial rise in P.D. of 31 to 33 mv., considerably less than the value, 53 mv., found in the measurement with the undiluted solution shown in Fig. 3, curve A. In five measurements with the 25-fold dilution, the change in P.D. was greater than with the fivefold dilution, the values varying from 36 to 60 mv., average, 50 mv. The P.D.-time curve marked C in Fig. 3 shows the results of an experiment with these two diluted RbCl-sea waters.

IV

Modified Sea Waters Containing NH_4Cl

The behavior of sea waters containing NH_4Cl proved to be very similar to that of the corresponding sea waters with RbCl, except that the changes in P.D. were even larger than those produced by either RbCl or KCl.

One experiment with NH_4Cl -sea water showed extremely large fluctuations in P.D., somewhat like those characteristic of KCl-rich solutions, but much more rapid. It was obvious that reliable values for the first maximum in such a P.D.-time curve could be obtained only with a measuring instrument of much shorter period than the

Compton electrometer used in these measurements. To avoid this difficulty, the present investigation was limited to solutions in which the concentrations of NH_4Cl were considerably smaller than 0.5 molar.

Nine measurements were carried out using 0.1 molar NH_4Cl in sea water. The best P.D.-time curve obtained in this series is shown as curve A in Fig. 4. The P.D.'s observed with these solutions were very high: in this instance, 87 mv. greater than the P.D. in ordinary sea water. As in the experiments with RbCl , the initial change in P.D. was marked, not by a maximum in the P.D.-time curve, but by a point of inflection, the position of which was often indefinite. In the curve shown in Fig. 4, the initial rise was about 68 mv. In three other curves where the position of the inflection point could be determined approximately, the rise in P.D. to this point varied between 20 and 53 mv.; the average of all four values was 44 mv.

When the sea water containing NH_4Cl was replaced by ordinary sea water, the P.D.-time curve (as shown in Fig. 4) closely resembled the curves obtained when KCl -rich solutions were followed by ordinary sea water.⁴⁶ Precisely the same behavior was observed in experiments with RbCl . The changes in P.D. (rapid fall to a minimum, rise to a maximum, followed by a slow fall) are just the reverse of the characteristic changes observed when K^+ enters the protoplasm from KCl -rich sea waters. It may accordingly be assumed that these changes in ordinary sea water are produced by K^+ , Rb^+ , or NH_4^+ coming out of the protoplasm. It is interesting to note that, in spite of differences in behavior on entering the protoplasm, all three ions exhibit the same type of P.D.-time curve when coming out.

Four measurements with 0.01 molar NH_4Cl in sea water showed rather large changes in P.D.: in one case as much as 25 mv. after 30 minutes. Similar results were obtained in three measurements with 0.001 molar NH_4Cl in sea water, where an increase of as much as 19 mv. was observed. With both solutions, however, the rise in P.D. was gradual, and did not permit assigning even an approximate value to the initial change.

The measurements with 0.1 molar NH_4Cl in sea water are therefore the only ones which provide data suitable for computing the apparent relative mobility of NH_4^+ in the *X* layer of *Valonia*. The same assumptions are made as in calculating the mobility of Rb^+ . If we take as the initial change in P.D. the value, 68 mv., from the experiment

shown in Fig. 4, the apparent mobility of NH_4^+ is found to be 108 times as great as that of Cl^- . If we use 44 mv. (the average of four discordant values) the mobility of NH_4^+ is found to be 37 times as great as that of Cl^- . In any case, it seems clear that the apparent mobility of NH_4^+ in the X layer is far greater than that of any of the other ions included in this study.

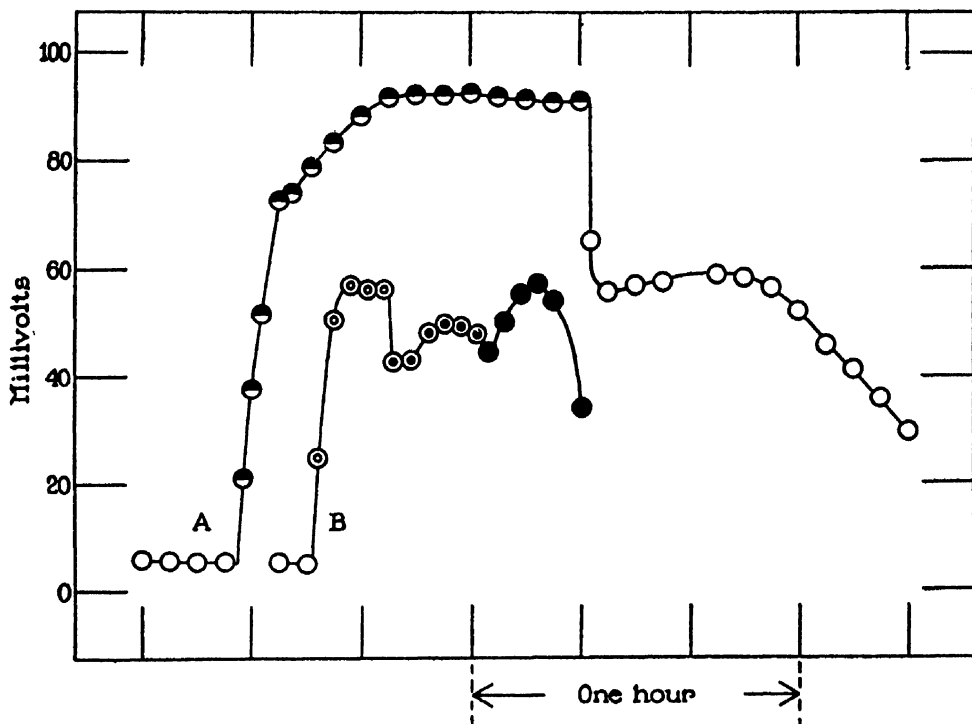


FIG. 4. Time curves showing changes in P.D. across *Valonia* protoplasm produced by solutions containing NH_4Cl . Curve A shows the changes when natural sea water (open circles) is replaced by 0.1 molar NH_4Cl in sea water (half-shaded circles) and when this solution in turn is replaced by natural sea water. Curve B shows the P.D.'s with three different dilutions of NH_4Cl -sea water: a 16.7-fold dilution, double circles, open centers; a fivefold dilution, double circles, shaded centers; and a 3.3-fold dilution, shaded circles. NH_4Cl -sea water contains 0.5 M NH_4Cl .

The high mobility of the NH_4^+ ion suggests that the concentration effect with NH_4Cl -sea water will be similar to the effects with KCl -sea water and RbCl -sea water. In particular, it is to be expected that the P.D.-dilution curve will pass through a minimum.

That this is true is shown in a qualitative manner by the experiment

reported in the P.D.-time curve B, in Fig. 4. It will be seen that the P.D. with the fivefold dilution of NH_4Cl -sea water is less than the P.D. with the 16.7-fold dilution which preceded it, and also less than the P.D. with the 3.3-fold dilution which followed it.

Such experiments, however, in which several dilutions of NH_4Cl -sea water are applied successively to the same cell, cannot be trusted to furnish quantitative data unless all the dilutions are greater than the critical value. Otherwise, NH_4^+ ions entering from one solution are liable to cause changes at inner layers of the protoplasm and affect the value of the P.D. with later solutions.

A number of additional measurements with these three diluted NH_4Cl -sea waters failed to furnish more quantitative information. Since all three dilutions (with the possible exception of 16.7) are evidently less than the critical value, NH_4^+ ions will enter the protoplasm and cause changes in P.D. at the inner layers. For quantitative comparisons, it is therefore necessary to determine the initial change in P.D. before NH_4^+ ions have diffused through the *X* layer. Unfortunately, the shapes of the P.D.-time curves usually obtained with these solutions gave no clew to the magnitude of this initial change in P.D.

DISCUSSION

The reason why the P.D.-time curves with sea waters containing RbCl or NH_4Cl do not rise sharply to well defined initial maxima can probably be traced to the greater speed with which these ions penetrate the protoplasm. If the curve is to show such a maximum, it is necessary that the entire¹⁰ outer surface of the protoplasm shall be brought into equilibrium with the new solution before the P.D.'s at inner layers of the protoplasm have been affected by the penetration of ions from the new solution. If the penetration of NH_4^+ (or Rb^+) is too rapid, however, it may not be possible to meet this requirement since an appreciable time is needed to leach out the cell wall and bring the whole surface of the protoplasm in contact with the new solution. In an extreme case, NH_4^+ ions might penetrate as far as the *Y* layer at one end of the cell before the NH_4Cl -sea water had reached the *X*

¹⁰ For a demonstration of the importance of applying the new solution to the entire outer surface of the cell, see Damon, E. B., *J. Gen. Physiol.*, 1932-33, 16, 376.

layer at the other end. The observed change in P.D. would then be the resultant of a very complex set of simultaneous changes, far too intricate to be interpreted.

If this explanation is correct, it should be possible to obtain P.D.-time curves with well defined maxima and minima (like the curves with KCl-sea water) by retarding the inward diffusion of NH_4^+ through the *X* layer of the protoplasm. This can be accomplished by employing dilute solutions (below the critical dilution) where the entrance of NH_4^+ is opposed, but not wholly prevented, by the potential gradient which arises from the outward diffusion of Na^+ and Cl^- from the vacuole. Fig. 5 shows the result of such an experiment, in which a threefold dilution of natural sea water was replaced by an equal dilution of a modified sea water containing 0.01 mole of NH_4Cl in a liter of the diluted solution. (Another such experiment, using fivefold dilutions, had exactly similar results.) It will be seen that the entrance of NH_4^+ from the diluted sea water led to a P.D.-time curve of precisely the same form as the curves resulting from the entrance of K^+ from undiluted KCl-rich sea waters. When the cell was returned to diluted natural sea water, the fluctuations in P.D. caused by NH_4^+ coming out of the protoplasm were just the reverse of those caused by NH_4^+ going in.

We may therefore conclude that sea waters with added KCl, RbCl, and NH_4Cl would all give P.D.-time curves of essentially the same form if these curves could be measured under ideal conditions. In such ideal experiments, the solutions would be applied instantaneously to the entire outer surface of the protoplasm, and the measuring instrument would be rapid enough to follow accurately all the fluctuations in P.D.

The greater speed with which Rb^+ and NH_4^+ enter the protoplasm is presumably the result of several contributing factors. Some of these will be discussed briefly.

For example, the concentration gradient in the protoplasm is doubtless greater for Rb^+ than for K^+ , since it may be expected that the protoplasm will normally contain a small concentration of K^+ , but will not contain any perceptible amount of Rb^+ . This somewhat steeper concentration gradient will be of greater importance when

the concentration of Rb in the external solution is small. The experiments with 0.1 molar RbCl in sea water furnish an example of the effect of small changes in the concentration gradient in the protoplasm. With cells which had been taken directly from ordinary sea water (and hence contained no Rb^+) the P.D.-time curves showed, at best, only a badly defined point of inflection. With cells which had been exposed for a short time to a sea water containing 0.05 molar RbCl,

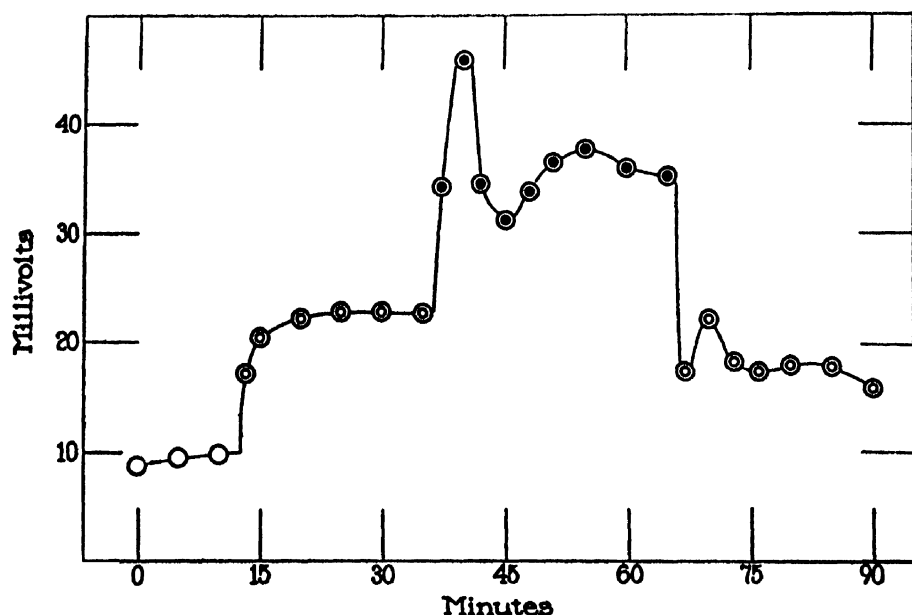


FIG. 5. P.D.-time curve showing the effect of substituting NH_4Cl for NaCl and KCl in diluted sea water. Open circles represent the P.D. in natural sea water; double circles with open centers, the P.D. in a threefold dilution of natural sea water; double circles with shaded centers, the P.D. in a threefold dilution of a modified sea water containing 0.01 mole of NH_4Cl in a liter of the diluted solution.

so that the protoplasm contained a little Rb^+ , treatment with 0.1 molar RbCl in sea water caused the P.D. to rise rapidly and pass through a well defined maximum. This difference in behavior may reasonably be attributed to the slower inward diffusion of Rb^+ in the second case, where the concentration gradient was less steep.

The speed with which NH_4^+ penetrates the protoplasm might be explained, in part, by the hypothesis that uncharged NH_3 molecules diffuse rapidly through the *X* layer, and on reaching the aqueous *W* layer react with water or some weak acid to form NH_4^+ ions.

The concentration effect with higher dilutions of NH_4Cl -sea water, however, must be considered as evidence against this hypothesis. With these dilute solutions, the potential gradient set up by the outward diffusion of Na^+ and Cl^- from the vacuole opposes the inward diffusion of NH_4^+ and other cations, but can have no effect on the diffusion of uncharged NH_3 molecules. Since it is found that the influence of NH_4Cl on the P.D. is greatly diminished (and probably finally annulled) when the external solution is diluted, we must conclude that it is principally the NH_4^+ ion, and not the NH_3 molecule, which enters the protoplasm from these NH_4Cl -rich solutions.

It is probable that differences in the partition coefficients for KCl , RbCl , and NH_4Cl (between water and the X layer) have a very important effect on the rates at which K^+ , Rb^+ , and NH_4^+ enter the protoplasm. Since the values of these coefficients are unknown, it has been assumed for purposes of calculation that the coefficients are all equal. It must therefore be emphasized that the numerical values obtained from these calculations represent merely the *apparent* relative mobilities of the ions.

It has been pointed out elsewhere¹⁶ that much higher dilutions of KCl -sea water than of natural sea water can be applied to the *Valonia* cell without at once producing certain secondary changes which are probably a sign of injury. It is interesting that equally high dilutions of RbCl -sea water or NH_4Cl -sea water (which do not contain potassium) may be applied to *Valonia* for a short time without causing these secondary changes. That is, these ions which resemble K^+ in their effect on the P.D. also exert the same protective action on *Valonia* protoplasm.

SUMMARY

In their influence on the P.D. across the protoplasm of *Valonia macrophysa*, Kütz., Li^+ and Cs^+ resemble Na^+ , while Rb^+ and NH_4^+ resemble K^+ . The apparent mobilities of the ions in the external surface layer of *Valonia* protoplasm increase in the order: Cs^+ , Na^+ , $\text{Li}^+ < \text{Cl}^- < \text{Rb}^+ < \text{K}^+ < \text{NH}_4^+$.

THE ULTRACENTRIFUGAL ANALYSIS OF THE LATENT MOSAIC VIRUS PROTEIN

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The virus protein that is the infectious principle of the latent mosaic disease of potatoes was first isolated by differential ultracentrifugation (1, 2) more than 2 years ago. It is much less stable than the corresponding protein of the tobacco mosaic disease; nevertheless, if sufficient care is taken its purified solution will yield a sharp centrifugal pattern indicative of an undamaged molecular species. Preparations also have been made by chemical procedures (3). Their properties have been in some ways different from those of the proteins isolated by ultracentrifugation. For this reason it has been important to see whether simple chemical treatments would lead to molecular alterations detectable in the analytical ultracentrifuge. The present paper records the results of several such observations together with a detailed study of the sedimentation pattern of this virus protein at a variety of pH values.

The writer is indebted to H. S. Loring for the samples used in this work. Parallel studies (4) of the biological activities and of virus properties of these samples have recently been described by him. Quantity ultracentrifugations were made in the same apparatus (5) that has been employed in all ultracentrifugal preparations (6) of this and other plant virus proteins. This machine carries heads about 7 inches in diameter and holds 150 cc.; the sedimentations necessary for the ultracentrifugal purification of the latent mosaic virus protein were effected by running the machine for an hour and a quarter at a speed of about 30,000 R.P.M. The analytical observations were carried out by the original absorption technique of Svedberg. The optimum centrifugal field

for such studies on the latent mosaic protein is about 30,000 times gravity.

For ultracentrifugal sedimentations the quantity heads were chilled in ice before use. The temperature of the head and its contained materials was never more than a few degrees above freezing at the conclusion of a run. Under such circumstances the virus protein of the latent potato disease can be repeatedly precipitated and resuspended in distilled water or dilute buffer

TABLE I
Sedimentation Constants of Latent Mosaic Virus Protein Solutions

pH	s_{20}	Time in buffer before being photographed	Remarks
3.3	114, 129	Immediate	Diffuse boundary
3.3		1 day	Complete precipitation
3.8	114, 137	Immediate	Diffuse
3.8	113	1 hr. then readjusted	Sharp
3.8	124, 141	20 hrs.	Very diffuse
4.1		1 day	Complete precipitation
5.7	112, 127	Immediate	Sharp; one preparation shows double boundary, other does not
6.1	111, 127	"	" "
7.0	112, 126	"	" "
7.0	109	3 days	Sharp
8.1	107	Immediate	"
9.8	113	"	"
10.1	109	"	"
10.1	109	36 hrs.	" unsedimentable
10.1	107	7 days	" "
10.3	Ca. 75, 115	Immediate	Moderately diffuse
11.0	<3	"	

solutions without suffering damage detectable with the analytical ultracentrifuge. The sedimentation diagram of a typical preparation obtained by subjecting the clarified juice of infected Turkish tobacco plants to four successive ultracentrifugations is shown in Fig. 1. The principal boundary is sharp; it moves at a rate corresponding to the constant $s_{20} = 113 \times 10^{-13}$ cm. sec.⁻¹ dynes⁻¹ (Table I). A faint sharp second boundary with s_{20} = about 130×10^{-13} can also be seen; the component causing this is some-

times absent, no matter whether the sample has been purified by ultracentrifugation or by salting-out.

Though repeated chemical precipitations lead to obvious damage of the virus protein, a single precipitation with either 30 per cent ammonium sulfate or potassium citrate followed by two ultracentrifugal sedimentations has given a sharp boundaried product if the salting-out was carefully carried out in the cold. Precipitation by HCl at pH 4 furnished a protein with a moderately sharp boundary. The protein precipitated by an equal volume of alcohol added at pH 5 was so thoroughly altered that it gave no measurable sedimenting boundary.

In previously reported investigations (7-9) sedimentation diagrams have been made of several virus protein solutions at a wide variety of pH values. These studies have demonstrated that in each instance the virus protein molecule and the infectivity of the virus have the same range of pH stability. This is to be expected if infectivity is a property of the intact virus protein molecule. The same type of study has now been made of the latent mosaic virus protein. Sedimentation diagrams have been prepared of solutions of the protein at pH values between 3.3 and 11 (Table I). Some of these diagrams have been obtained immediately after pH adjustment, others after standing for various lengths of time. These results should be compared with the pH inactivation studies of Loring (4). Such a comparison makes it clear that this virus protein molecule too is stable within the same general limits that define its virus activity. The same constant, within experimental error, is observed from all preparations at pH values between about 5.7 and 10.1. On the alkaline side of pH 10.1 the breakdown is practically immediate and complete, in contrast to the behavior of the tobacco mosaic virus protein. At pH 10.3 a more rapidly moving boundary with $s_{20} =$ about 75×10^{-13} appears (Fig. 5); the boundaries of solutions standing at this pH rapidly became so diffuse that accurate measurements were impossible. Slightly greater alkalinity (pH 10.7 to 11.0) leaves fragments that sediment no faster than the molecules of egg albumin (Fig. 6). In acid affairs are not so simple, because the limit of stability is not far from the isoelectric point of the protein (at pH 4). In the range from about pH 4 to 5 not enough of the protein is in solution to provide a boundary. At pH 3.8

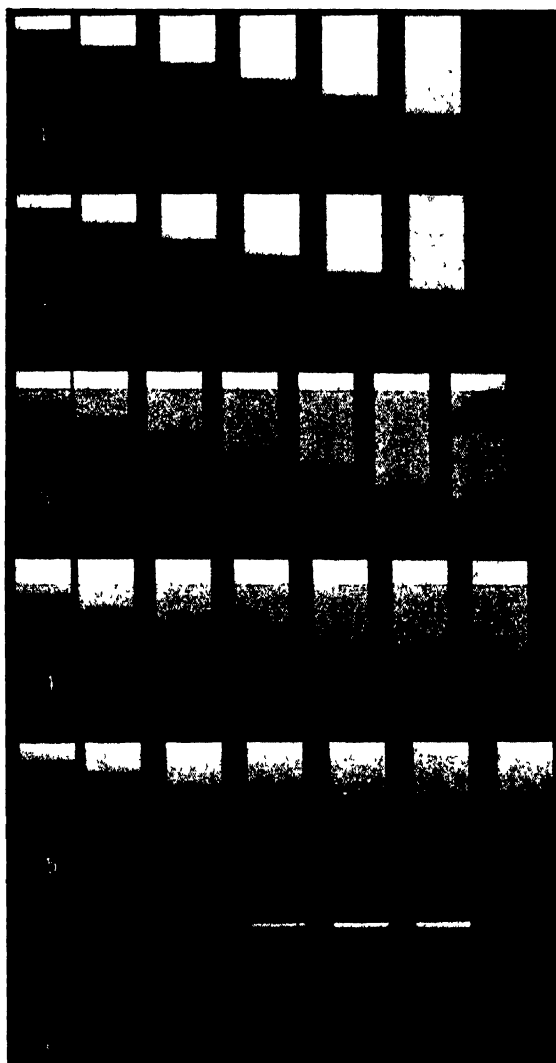


FIG. 1. Sedimentation diagram of a sample of the latent mosaic virus protein purified by a 4-fold quantity ultracentrifugation with distilled water as resuspending fluid. In this and each of the following diagrams the interval between exposures was exactly 5 minutes and the mean centrifugal field about 31,000 times gravity.

FIG. 2. Diagram of the virus protein in pH 5.0 buffer immediately after pH adjustment.

FIG. 3. Diagram of the virus protein made immediately after adjustment to pH 3.8.

FIG. 4. Diagram of the virus protein after being held for 1 hour at pH 3.8 and then readjusted to pH 7.

FIG. 5. Diagram of the virus protein made immediately after adjustment to pH 10.3.

FIG. 6. Diagram of the virus protein made immediately after adjustment to pH 11.0.

the usual boundaries can be photographed but they are only moderately sharp immediately after pH adjustment (Fig. 3) and a day later are very diffuse. That the molecular change responsible for this diffuseness is reversible and perhaps due to associations in the neighborhood of the isoelectric point is indicated by the fact that if the pH is readjusted to neutrality after a short time at pH 3.8, a sharp pattern is once more obtained (Fig. 4). Loring found (4) that such readjusted solutions had their full activities. A photograph made immediately after adjustment to pH 3.3 also shows a diffuse boundary but after a day at this acidity precipitation is complete and no boundary can be observed.

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STUDIES ON A BACTERICIDAL AGENT EXTRACTED FROM A SOIL BACILLUS

I. PREPARATION OF THE AGENT. ITS ACTIVITY IN VITRO

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Microorganisms perform a vast number of biochemical reactions, many of which are not known to occur in the animal and plant kingdoms (1). On the basis of present knowledge it is conceivable that one may find in nature microbial species endowed with catalysts capable of activating almost any type of biochemical reaction. During the past few years, this point of view has found its application in the isolation of soil microorganisms which selectively attack certain substances of interest to the biochemist (2) and to the immunologist (3-8). It may be recalled in particular that soluble polysaccharides, extracted from several bacterial pathogens, have been found to be decomposed by certain microbial species, although the same substances are resistant to the action of all known enzymes of animal and plant origin.

It appeared possible that there also exist in nature microorganisms capable of attacking not only isolated soluble components of other bacterial cells, but also the intact living cells themselves. Actually we have isolated from soil a spore-bearing bacillus which attacks and lyses the living cells of several species of Gram-positive microorganisms. The present paper describes the isolation of this new soil bacillus, and the preparation, properties, and activity of the soluble agent by means of which it attacks and lyses the living cells of the susceptible, Gram-positive species.

EXPERIMENTAL

Isolation of a Sporulating Bacillus Capable of Lysing the Living Cells of Gram-Positive Microorganisms.—The method employed for the discovery of microorganisms capable of attacking certain definite organic compounds has already been described (2, 3). It is based on the assumption that all organic matter added to the soil eventually undergoes decomposition through the agency of microorganisms. In the present case, it was hoped

that the addition to soil of living cultures of Gram-positive cocci would result in the development of a selective soil flora capable of attacking the living cells of these bacterial species.

Soil samples of neutral reaction, obtained from a number of different sources, were pooled, and mixed with alkaline phosphate, ammonium sulfate, and an excess of calcium carbonate. The mixed sample was kept at about 70 per cent of its moisture-holding capacity and incubated at 30°C. for a few weeks in order to bring about the decomposition of most of the organic matter originally present.

Staphylococci, pneumococci (R variants), and group A hemolytic streptococci (glossy variants) were grown in beef infusion peptone broth and centrifuged; the living cells, resuspended in small volumes of distilled water, were added to the soil sample. After two years, during which this process was repeated at irregular intervals of time, a small amount of the soil preparation (2 gm.) was added to 10 cc. of mineral medium ($M/20 K_2HPO_4$, $M/30 NaH_2PO_4$, $M/100 (NH_4)_2SO_4$, tap water) to which had been added the living cells recovered from 150 cc. of staphylococcus culture. This suspension, containing soil and staphylococci, was incubated in shallow layers at 30°C. After 48 hours incubation, stained films of the bacterial suspension revealed advanced lysis of the staphylococci and the presence of a mixed bacterial flora. A small amount of the same suspension was now transferred to a similar medium and incubated under the same conditions. Again, lysis of the staphylococci was observed within 48 hours. A few more transfers were sufficient to eliminate most of the microbial species originally present in the soil used as inoculum. By plating on peptone agar, it was finally possible to isolate in pure culture a Gram-positive bacillus capable of lysing living staphylococci resuspended in the mineral medium described above.

Description of the Organism.—The organism is a motile spore-bearing bacillus measuring in average $4 \times 0.5 \mu$. The spores are terminal, oval; they resist heating at 80°C. but are killed at boiling temperature.

Very young cells of the bacillus retain the Gram stain, but they soon lose this characteristic and appear then as Gram-negative rods. This change in staining reaction is probably associated with the fact that cultures undergo autolysis very rapidly (24 to 48 hours), giving rise to shadow cells with intensely stained granules, Gram-negative detritus, and a mass of oval spores.

The organism grows readily and abundantly on peptone media, especially when incubation is carried out in shallow layer at 37°C. No gas is formed in sugar peptone media, and the culture does not become acid (the following substrates were tested; arabinose, dextrose, dulcitol, inulin, lactose, mannose, mannitol, rhamnose, saccharose, salicin, sorbitol, xylose). Litmus milk becomes alkaline. The fact that indicators do not reveal the production of acid does not necessarily mean, however, that the substrates are not fermented by the culture. It has been observed that the growth of the bacillus in peptone solution renders the medium very alkaline (pH 9.0) and it is possible that the production of alkaline substances may mask the production of organic acids from the sugars added to the peptone.

The organism liquefies gelatin and gives a diffuse zone of hemolysis on blood agar plates. It gives a negative V.P. test and forms H_2S on lead acetate agar medium. Catalase production is very abundant.

Preparation and Properties of a Soluble Bacterial Extract Which Attacks the Living Cells of Gram-Positive Microorganisms.—As stated above, the spore-bearing bacillus just described is capable of lysing suspensions of living staphylococci. The lysis is caused by an agent which is found in solution in autolysates of cultures of the soil bacillus. When very young cultures (12 to 18 hours) of the bacillus are used, the active agent is found in the bacterial bodies; it can then be obtained in solution by collecting the bacterial cells, and allowing them to autolyze in an aqueous medium. As the culture becomes older, however, more and more of the active material is found in solution in the culture medium and, after 72 hours (when practically all cells have autolyzed) only a small percentage of the activity is found associated with the cellular material.

The active principle is quantitatively precipitated at pH 4.5; the precipitate, redissolved at neutral reaction, exhibits the activity of the original solution.

On the basis of these observations, the following method has been employed for the preparation and concentration of the active extract used in the experiments described in this and the following paper.

The organism is grown in a medium consisting of 1 per cent acid hydrolysate of casein in tap water, at pH 7.0. The culture is incubated in shallow layer (3 cm.) for 3 to 4 days at 37°C. At the end of the incubation period, stained films of the culture show the presence of Gram-negative shadow forms, and of large numbers of spores, and the reaction of the medium has become alkaline (pH 9.0). The culture is then centrifuged for 1 hour at 3,500 R.P.M. The clear supernatant fluid is separated, and acidified to pH 4.5 (this requires about 4 cc. of concentrated HCl per liter of autolysate). A faint precipitate forms; it is separated by centrifugation or filtration and redissolved at neutral reaction.

Work is now in progress toward further purification of the active material; a complete description of its properties and composition is therefore reserved for the future, when pure preparations are available. It may be stated, however, that the preparations obtained by the technique just described give all the protein tests and contain 14.5 per cent nitrogen. The active principle does not dialyze through collodion membranes; it retains its activity after 10 minutes heating at 90°C. at pH 2.0 and pH 9.0. It is also resistant to the action of pepsin, of crystalline trypsin and chymotrypsin, and of crude trypsin. It must be pointed out, however, that although treatment with heat or with proteolytic enzymes does not inactivate the bactericidal agent, it renders it insoluble in neutral buffer solutions.

Filtration through Berkefeld candles (V) gives preparations which are capable of lyzing the susceptible bacterial species although much activity is lost during the process. Filtration through Chamberland filters or asbestos pads has always resulted in complete loss of activity.

The Effect of the Soluble Bacterial Extract upon the Living Cells of Several Microbial Species.—The effect of the bacterial extract upon different microbial species has been studied under a variety of conditions which will be described in this and other publications. It is permissible to state at the present time, however, that the bacterial extract has been found to exert a marked bactericidal effect upon all the Gram-positive species thus far tried, whereas all the Gram-negative bacilli have remained unaffected. Gram-negative cocci and acid-fast bacilli have not yet been tried.

The following microbial species have been studied:

A. Gram-positive species (all susceptible to the bactericidal effect of the extract). *Diplococcus pneumoniae*: Five strains of virulent encapsulated pneumococci (Types I, II, III, V, and VIII). Three strains of avirulent rough pneumococci (derived from Types I, II, III). *Streptococcus hemolyticus*: Eight matt and glossy strains of group A (types 1, 3, 6, 14, 20). Three virulent strains of group C. Three strains of group D (cheese streptococci). *Streptococcus viridans*: Two strains. Indifferent (gamma) streptococci: Two strains. *Staphylococcus aureus*: One rabbit virulent strain, one mouse virulent strain. Unidentified culture of spore-bearing Gram-positive bacillus. *Saccharomyces cerevisiae*.

B. Gram-negative species (not susceptible to the bactericidal effect of the extract). *Escherichia coli*. *Eberthella typhi*. *Salmonella paratyphi*. *Klebsiella pneumoniae* type B. *Hemophilus influenzae*. Unidentified culture of spore-bearing Gram-negative bacillus.

The activity of the bacterial extract upon the cells of the different microbial species, as measured by lysis and loss of viability, was studied in the following experiments.

The test cultures were grown for 8 hours in meat infusion peptone broth; the cells were separated by centrifugation and resuspended in phosphate buffer (pH 7.6) to give suspensions containing about 10^8 cells per cc. The suspensions, distributed in 1 cc. amounts into test tubes, were treated with different amounts of bacterial extract (preparation NS7) and made up to a final volume of 2 cc. with phosphate buffer (see Table I).

After 3 hours incubation at 37°C. the different preparations were observed for the occurrence of lysis (turbidity readings, confirmed by microscopic examinations), and streaked on blood agar plates to determine the effect of the bacterial extract on the viability of the cells (Table I).

TABLE I
Effect of a Soluble Bacterial Extract on Different Microbial Species

Test organisms		Amount of extract used (mg. per 10 ⁹ cells)				
		5	1	0.1	0.01	0
<i>Diplococcus pneumoniae</i> (8 strains)	Lysis*	C	C	C	P	N
	Viability†	—	—	—	—	++++
	Reductase‡	NR	NR	NR	NR	CR
<i>Streptococcus hemolyticus</i> group A (7 strains)	Lysis	P or N	N	N	N	N
	Viability	—	—	—	—	++++
	Reductase	NR	NR	NR	NR	CR
<i>Streptococcus hemolyticus</i> group C (3 strains)	Lysis	P or N	N	N	N	N
	Viability	—	—	—	—	++++
	Reductase	NR	NR	NR	NR	CR
<i>Streptococcus hemolyticus</i> group D (3 strains)	Lysis	N	N	N	N	N
	Viability	—	+	++++	++++	++++
	Reductase	NR	PR	CR	CR	CR
<i>Streptococcus viridans</i> (2 strains)	Lysis	P or N	N	N	N	N
	Viability	—	—	—	—	++++
Indifferent streptococcus (2 strains)	Lysis	N	N	N	N	N
	Viability	—	—	—	—	++++
<i>Staphylococcus aureus</i> (2 strains)	Lysis	C	C	P	N	N
	Viability	—	—	—	++++	++++
	Reductase	NR	NR	NR	CR	CR
Gram-positive spore-bearing bacillus	Lysis	X	C	X	X	N
<i>Escherichia coli</i>	Lysis	N	N	N	N	N
	Viability	++++	++++	++++	++++	++++
	Reductase	CR	CR	CR	CR	CR
<i>Klebsiella pneumoniae</i> group B	Lysis	N	N	N	N	N
	Viability	++++	++++	++++	++++	++++
	Reductase	CR	CR	CR	CR	CR
<i>Hemophilus influenzae</i>	Lysis	N	N	N	N	N
	Viability	++++	++++	++++	++++	++++
Gram-negative sporulating bacillus	Lysis	X	N	X	X	N
	Viability	X	++++	X	X	++++
<i>Saccharomyces cerevisiae</i>	Lysis	N	N	N	N	N
	Viability	—	++++	++++	++++	++++
	Reductase	NR	CR	CR	CR	CR

* C = complete lysis. P = partial lysis. N = no lysis.

† — = no growth on blood agar. + = much reduced growth on blood agar.
++++ = abundant growth on blood agar.

‡ CR = complete reduction of the methylene blue. PR = partial reduction of the methylene blue. NR = no reduction of the methylene blue. X = not done.

The results presented in Table I indicate that the soluble bacterial extract exerts a bactericidal effect on all the Gram-positive microorganisms so far tested. Unequivocal evidence of lysis was recognized only in the case of pneumococci, staphylococci, and a Gram-positive spore-bearing rod. The soluble extract exerted no bactericidal effect, nor did it cause the lysis of any of the Gram-negative species.

The bactericidal effect of the extract was concluded from the inability of the treated susceptible cells to grow on subsequent transfer to blood agar plates. In other tests, these results were confirmed by inoculating the cells into liquid media, where they failed to multiply, or by injecting the virulent species into susceptible animals, which they failed to kill.

It is worth emphasizing the comparative effects of different amounts of extract in causing the death or the lysis of the various microorganisms. In the case of pneumococci, staphylococci, and the Gram-positive spore-bearing rods, death of the cell is accompanied by lysis. On the contrary, streptococci do not lyse although they are susceptible to the bactericidal effect of the extract; in fact, no lysis of the streptococci is observed even when they are treated with 100 times the minimal amount of extract required to kill the cells. It is also worth noting that staphylococci, although much more resistant than group A hemolytic streptococci to the killing effect of the extract, undergo lysis very readily.

It appears therefore that no parallelism exists between lytic effect and bactericidal effect; in fact, it will be shown later in this article that lysis is only a secondary process, caused by the action of the autolytic enzymes of the cells, and follows some other primary injury inflicted by the bactericidal agent.

Several attempts have been made to compare the effect of the bactericidal agent upon pneumococci and upon group A hemolytic streptococci in different culture phases (rough and smooth, glossy and matt variants). This was studied by determining the minimal amount of extract required to kill the same number of cells of different species in a given time, or by measuring the length of time required by a same amount of extract to kill the same number of cells. Under these conditions no difference could be found between R and S pneumococci, or between glossy, matt avirulent, and matt virulent streptococci, irrespective of type derivation. Furthermore pneumococci, group A and group C hemolytic streptococci, green and indifferent streptococci, were all found to be equally susceptible to the bactericidal agent.

The Inhibitory Effect of the Bactericidal Agent on the Glucose Dehydrogenase of Microorganisms.—The results presented in Table I indicate that the

minimal effective dose of the bactericidal agent is not the same for all the microbial species. Yeast and cheese streptococci require the largest amount (1 to 5 mg. per 10^9 cells), next come the staphylococci (0.1 mg. per 10^9 cells); the pneumococci, hemolytic streptococci of group A and C, green and indifferent streptococci, are all killed by very small amounts of extract (0.01 mg.). In a general way, it can be said that the resistance of the various microbial species to the bactericidal agent varies in the order of their metabolic activity, as measured by their ability to reduce methylene blue in the presence of glucose: yeast and cheese streptococci (group D) metabolize more actively than staphylococci, which in their turn are more active than pneumococci and hemolytic streptococci of group A. This parallelism suggested that the primary toxic effect of the bactericidal agent might be directed against the dehydrogenase system of the microbial cell; some preliminary experiments were instituted to test this point.

Microbial suspensions containing approximately 10^9 cells per cc. were used. They were distributed in 3 cc. amounts into test tubes and were treated with different amounts of the bactericidal agent (Table I). The mixtures were incubated for 3 hours at 37°C . Methylene blue (1 cc. of 0.002 M solution) and glucose (1 cc. of 10 per cent solution) were then added, and the mixtures, sealed with vaseline, were incubated at 37°C . The rate of reduction of the dye was observed. Although the data concerning the experimental procedure, and the results obtained, will be presented *in extenso* in another publication, a general summary of the final results is incorporated in Table I.

It is clear that, in the case of all the Gram-positive organisms, incubation of the microbial cells with sufficient amounts of the bactericidal extract results in an inhibition of methylene blue reduction. The glucose dehydrogenase of the Gram-negative bacilli, on the contrary, is not affected by the same treatment.

In all cases, the minimal amount of bactericidal extract required to kill the microbial cells is also sufficient to inhibit their reducing action. In other words, the loss of viability appears to be quantitatively related to the inactivation of the glucose dehydrogenase of the cell.

Inhibition of Growth by the Bactericidal Agent.—The lytic effect of the soluble agent upon pneumococci and staphylococci, and its bactericidal effect upon Gram-positive organisms in general, can be observed not only when the susceptible cells are resuspended in buffer solution, but also in the presence of meat infusion, peptone, serum, and ascitic fluid. It was to be expected, therefore, that the extract would inhibit the growth of the susceptible species in culture media.

Inhibition of growth has in fact been observed in the case of all the Gram-positive organisms mentioned in Table I. A single example will illustrate

the activity of the extract in this respect. Test tubes containing 5 cc. of meat infusion peptone broth were inoculated with 0.03 cc. of a pneumococcus culture (D39R), and treated with different amounts of bactericidal agent (preparation NS7). Full growth had developed within 12 hours in the untreated control tubes whereas it took 24 hours for growth to appear in the tube which had received 0.000,01 mg. of extract. No growth developed in the tubes which received 0.000,1 mg. (or more) of the same preparation. On the contrary, no inhibition or retardation of growth was observed with any of the Gram-negative bacilli; for instance *Escherichia coli*, *Klebsiella pneumoniae*, *Eberthella typhi*, grow normally in the presence of large amounts of extract, even when an inoculum as small as 10^{-7} cc. is used.

DISCUSSION

The bactericidal agent described in the present paper is associated with a protein fraction which precipitates out of solution at pH 4.5. Heating at 90°C., or digestion with proteolytic enzymes, renders the active fraction insoluble, but does not in any way affect its lytic or bactericidal power, as can be shown by adding the suspension of insoluble material to the susceptible bacterial species. It is possible therefore, that the active substance itself is not a protein, but that the protein with which it is associated determines its solubility properties.

Although the agent exerts a bactericidal effect on the cells of all the Gram-positive species so far tested, its lytic effect has been observed only against pneumococci, staphylococci, and an unrelated Gram-positive spore-bearing bacillus. It is likely, therefore, that the death of the cell does not result from a lytic action of the extract, but that on the contrary, lysis is only a secondary process. The bacteriological literature offers several examples of "secondary lysis" following treatment with various antiseptics (heavy metals, formaldehyde, iodine, bile salts, toluol, acetone, etc.) (9, 10). In fact, the "bile solubility" of pneumococci is a good example of this phenomenon. Bile salts, unsaturated fatty acids, do not by themselves lyse the pneumococci; they inflict upon the cell an injury which destroys some essential metabolic function, without at the same time destroying the autolytic enzymes; the autolytic system, held in abeyance in the normal living cell, then begins to function, and autolysis follows (11, 12).

It is worthy of notice that group A hemolytic streptococci are known to be very resistant to normal autolysis; staphylococci autolyze more rapidly and pneumococci most rapidly of all. It has now been found that the

bactericidal agent considered in the present paper "lyzes" pneumococci most readily, staphylococci somewhat more slowly, and streptococci little or not at all, even though group A hemolytic streptococci are as susceptible as pneumococci to the bactericidal effect of the agent. Furthermore, it has been found that the bactericidal agent does not lyse the cells of pneumococci or staphylococci in which the autolytic enzymes have been destroyed by heating, or by treatment with formaldehyde. All these facts, when taken together, suggest that the bactericidal agent exerts a toxic action upon the living cells of the susceptible species and that lysis, when it occurs, is only a secondary process, caused by the cells' own autolytic enzymes.

How then does the bactericidal agent exert its toxic effect? It has been found that the minimal amount of extract which causes the death of the cell also destroys its ability to reduce methylene blue in the presence of glucose. This is not, however, sufficient evidence to establish that the inhibition of the glucose dehydrogenase is the cause of cell death. It remains possible that both effects are the common result of some other primary injury, as yet unrecognized. In the analysis of this question, it may be important to keep in mind that the minimal amount of extract required to kill a given number of cells is the same whether the test is carried out with pneumococci, or hemolytic streptococci of group A and C, or green and indifferent streptococci, irrespective of type derivation and culture phase (rough and smooth, matt and glossy variants). This observation suggests that the effect of the bactericidal agent is directed against a cellular structure or function which is common to all these bacterial species.

Furthermore, it is worth emphasizing again that the extract appears to be effective only against Gram-positive microorganisms. No lytic or bactericidal effect, no inhibition of glucose dehydrogenase, no retardation of growth could be observed with any of the Gram-negative bacilli so far tested (Gram-negative cocci and acid-fast bacilli have not yet been tested). Many examples are already known of differential toxic action of antiseptics upon Gram-positive and Gram-negative species (13-15). In fact, the Gram stain appears to divide the microbial world into two groups which differ widely, not only in several of their physiologic properties, but also in the chemical structure of the cell (16). Professor Christian Gram, who introduced in bacteriology the staining technique which bears his name, died recently (17). It may be proper at this time to suggest that the Gram reaction, which has proved of such great importance in the identification and classification of microbial species, may in the future serve as a guide in the study of fundamental problems of bacterial physiology and cytology.

SUMMARY

A Gram-positive, spore-bearing, aerobic bacillus, capable of lyzing the living cells of many Gram-positive microbial species, has been isolated from soil.

Cultures of this soil bacillus in peptone media release during autolysis a soluble agent which exerts a bactericidal effect on all the Gram-positive microorganisms so far tested, and inactivates their glucose dehydrogenases. It also inhibits the growth of the susceptible species in culture media.

Several of the Gram-positive species undergo lysis when incubated with the bactericidal agent. It appears however, that lysis is only a secondary process, due to the autolytic enzymes of the susceptible cells, and that it follows upon some other primary injury caused by the active agent.

The bactericidal agent is ineffective against all the Gram-negative bacilli so far tested.

Addendum.—The bactericidal agent described in the present paper has now been obtained in a form free of protein; the new purified preparations are about 50 to 100 times more active, both *in vitro* and *in vivo*, than the ones used in the experiments which have just been recorded.

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STUDIES ON A BACTERICIDAL AGENT EXTRACTED FROM A SOIL BACILLUS

II. PROTECTIVE EFFECT OF THE BACTERICIDAL AGENT AGAINST EXPERIMENTAL PNEUMOCOCCUS INFECTIONS IN MICE

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In the preceding paper a description was given of the preparation and properties of a soluble agent, extracted from cultures of an unidentified soil bacillus, which exerts a bactericidal effect on Gram-positive microorganisms (1). The bactericidal effect *in vitro* is not inhibited by the presence of serum or ascitic fluid; in fact, the bactericidal agent is also effective *in vivo*. Its protective action against experimental pneumococcus infections in mice is described in the present paper.

EXPERIMENTAL

Cultures.—Virulent pneumococci of Types I, II, III, V, VIII, and virulent Friedländer bacilli type B were grown in blood broth and passed through mice often enough to maintain a degree of virulence such that 0.000,000,01 cc. of an 8 hour culture would regularly kill 20 gm. mice within 72 hours. In all cases, the infective dose, diluted in 0.5 cc. of buffer pH 7.4, was injected by the intra-abdominal route.

Bactericidal Agent.—All the protection experiments reported in the present paper were carried out with the same preparation of the bactericidal agent (NS7) which was used in the experiments described in the preceding paper. The agent was injected by the intra-abdominal route in the form of a solution in phosphate buffer at pH 7.4. The dilutions were so arranged that the desired amount of agent was administered in 0.5 cc. of buffer solution.

Protective Effect of a Single Dose of the Bactericidal Agent.—Mice were infected with varying dilutions of cultures of virulent pneumococci (Type I and Type III) and were subsequently treated within 10 minutes with 2 mg. of the bactericidal agent. No further treatment was given (Table I).

The results presented in Table I show that one single treatment with 2 mg. of the bactericidal substance was sufficient to protect a significant number of mice against infection with 0.0001 and 0.00001 cc. of culture of virulent pneumococci. Although most of the mice infected with larger amounts of culture died within the 6 day observation periods, they sur-

vived longer than the untreated controls. The next experiment aimed at determining whether a higher degree of protection could be obtained by administering the bactericidal extract repeatedly at 24 hour intervals.

Protective Effect of Repeated Doses of the Bactericidal Agent.—Mice were inoculated with 0.1 or 0.01 cc. of cultures of virulent pneumococci Types I, II, III, V, VIII. They were treated on 3 consecutive days; 2 mg. of the bactericidal agent was given 10 minutes after injection of the infective inoculum, 1 mg. 24 hours later, and 1 mg. 48 hours later (Table II.)

TABLE I
Protective Effect of a Single Dose of Bactericidal Substance

Infective dose of pneumococcus		Treat- ment	Number of mice	Result*					
	cc.	mg.							
Type I	0.01	2	6	D 48	D 72	D 72	D 72	D 72	S
"	0.001	2	6	D 72	D 72	D 96	S	S	S
"	0.000,1	2	6	D 72	S	S	S	S	S
"	0.000,01	2	6	S	S	S	S	S	S
"	0.000,001	0	1	D 44					
"	0.000,000,1	0	1	D 46					
"	0.000,000,01	0	1	D 72					
Type III	0.01	2	6	D 48	D 48	D 72	D 72	D 72	D 72
"	0.001	2	6	D 72	D 72	D 72	D 96	D 96	D 96
"	0.000,1	2	6	D 72	D 96	D 96	S	S	S
"	0.000,01	2	6	D 72	D 96	S	S	S	S
"	0.000,001	0	1	D 46					
"	0.000,000,1	0	1	D 44					
"	0.000,000,01	0	1	D 44					

*S = survival of the animal (6 day observation periods).

D = death of the animal; the numeral indicates number of hours before death.

The results presented in Table II show that, with 3 consecutive treatments at 24 hour intervals of time and comprising in all 4 mg. of agent, it was possible to protect many mice against 0.1 and 0.01 cc. of cultures of pneumococci of maximum virulence; in all cases the untreated control mice, inoculated with 0.000,000,01 cc. of culture or with larger infective doses, died in less than 72 hours.

In order to study the comparative effectiveness of the bactericidal agent against the different types of pneumococci, an effort was made in the following experiment to determine the minimal amount of agent that would protect mice against the same infective dose of pneumococci of different types.

Titration of the Bactericidal Agent against Different Types of Pneumococci.—Groups of 4 mice each were inoculated with 0.001 cc. of 8 hour cultures of pneumococci of Types I, II, III, V, VIII. Within 10 minutes after inoculation each group was treated with 1 mg., 0.3 mg., or 0.1 mg. respectively of the bactericidal agent. A second treatment with the same amount of extract (1 mg., 0.3 mg., and 0.1 mg. respectively) was again given 24 hours later and the same dose repeated 48 hours after inoculation (Table III).

TABLE II
Protective Effect of Repeated Treatments with the Bactericidal Agent

Infective dose of pneumococcus		Treatment on 3 consecutive days			Number of mice	Results					
	cc.	mg.	mg.	mg.							
Type I	0.1	2	1	1	6	D 22	S	S	S	S	S
"	0.01	2	1	1	6	D 22	S	S	S	S	S
"	0.000,000,01	0	0	0	1*	D 40					
Type II	0.1	2	1	1	6	D 26	S	S	S	S	S
"	0.01	2	1	1	6	D 22	S	S	S	S	S
"	0.000,000,01	0	0	0	1*	D 72					
Type III	0.1	2	1	1	6	D 72	D 72	D 72	D 72	S	S
"	0.01	2	1	1	6	D 72	D 72	S	S	S	S
"	0.000,000,01	0	0	0	1*	D 60					
Type V	0.1	2	1	1	6	D 72	S	S	S	S	S
"	0.01	2	1	1	6	S	S	S	S	S	S
"	0.000,000,01	0	0	0	1*	D 72					
Type VIII	0.1	2	1	1	6	D 22	D 22	D 40	D 48	S	S
"	0.01	2	1	1	6	D 22	S	S	S	S	S
"	0.000,000,01	0	0	0	1*	D 40					

* The untreated control animals inoculated with 0.000,000,1 and 0.000,001 cc. of culture died within 44 hours.

It is apparent from the results presented in Table III that 1 mg. of extract administered daily on 3 consecutive days was sufficient to protect mice against infection with 0.001 cc. of culture of pneumococci Types I, II, III, V, VIII. When the amount of extract was reduced to 0.3 mg. or 0.1 mg. daily, most animals died within the 6 day observation period, although they survived longer than the controls. One may conclude therefore, that the protective effect of the bactericidal agent is approximately the same against all virulent pneumococci so far tested, irrespective of specific type.

In the three experiments which have just been described, the first dose

of bactericidal agent was administered within 10 to 15 minutes after inoculation of the experimental animals with the infecting organism. In the following experiment an attempt was made to determine the curative effect of the bactericidal agent when administered several hours after injection of the infecting inoculum.

TABLE III
Titration of the Bactericidal Agent against Pneumococci of Different Types

Infective dose of pneumococcus		Treatment			Number of mice	Results			
	cc.	mg. of agent per day							
Type I	0.001	1.0	1.0	1.0	4	S	S	S	S
"	"	0.3	0.3	0.3	4	D 72	D 96	D 120	S
"	"	0.1	0.1	0.1	4	D 72	D 72	D 96	D 96
"	"	0	0	0	4	D 24	D 24	D 48	D 48
Type II	0.001	1.0	1.0	1.0	4	S	S	S	S
"	"	0.3	0.3	0.3	4	D 72	D 96	D 96	D 120
"	"	0.1	0.1	0.1	4	D 72	D 72	D 96	D 96
"	"	0	0	0	4	D 24	D 24	D 48	D 48
Type III	0.001	1.0	1.0	1.0	4	D 120	S	S	S
"	"	0.3	0.3	0.3	4	D 72	D 96	D 96	D 96
"	"	0.1	0.1	0.1	4	D 48	D 72	D 72	D 72
"	"	0	0	0	4	D 24	D 24	D 48	D 48
Type V	0.001	1.0	1.0	1.0	4	S	S	S	S
"	"	0.3	0.3	0.3	4	D 96	D 120	D 120	S
"	"	0.1	0.1	0.1	4	D 72	D 72	D 96	D 96
"	"	0	0	0	4	D 48	D 48	D 48	D 48
Type VIII	0.001	1.0	1.0	1.0	4	D 120	S	S	S
"	"	0.3	0.3	0.3	4	D 72	D 96	D 96	D 96
"	"	0.1	0.1	0.1	4	D 48	D 48	D 72	D 72
"	"	0	0	0	4	D 24	D 24	D 24	D 24

The Curative Effect of the Bactericidal Agent.—Mice were inoculated with 0.000,01 cc. of culture of Type I or Type III. They were divided into three groups which were treated with 2 mg. of bactericidal agent respectively 2 hours, 5 hours, and 17 hours after infection. A second and a third dose of 1 mg. each, were given 24 and 48 hours after the first treatment (Table IV).

The results presented in Table IV show that mice inoculated with 1000 fatal doses of pneumococci Type I or Type III can be protected even when treatment with the bactericidal agent is delayed for several hours after

infection. In fact, 3 out of 6 mice inoculated with Type I organisms survived, although treatment had been delayed for 17 hours; the 6 mice inoculated with Type III pneumococci and treated 17 hours later all died, but 4 of them survived much longer than the untreated controls.

The Effect of the Bactericidal Agent upon Infection with Klebsiella pneumoniae.—It has been shown in the preceding paper that the bactericidal agent is ineffective *in vitro* against all the Gram-negative bacilli so far tested. It is also ineffective upon experimental infection of mice with

TABLE IV
Curative Effect of the Bactericidal Agent

Infective dose of pneumococcus		Time between infection and first treatment			No treatment
		2 hrs.	5 hrs.	17 hrs.	
Type I	0.000,01	S	S	D 34	D 30
"	"	S	S	D 72	D 34
"	"	S	S	D 96	D 40
"	"	S	S	S	—
"	"	S	S	S	—
"	"	S	S	S	—
"	0.000,000,01	—	—	—	D 72
Type III	0.000,01	D 96	D 72	D 24	D 18
"	"	S	D 96	D 30	D 30
"	"	S	S	D 48	D 34
"	"	S	S	D 60	—
"	"	S	S	D 72	—
"	"	S	S	D 96	—
"	0.000,000,01	—	—	—	D 48

Klebsiella pneumoniae (Friedländer bacillus), a Gram-negative rod, as appears from the following experiment.

Mice were inoculated with dilutions of a 6 hour culture of Friedländer bacilli type B. They were treated within 5 minutes with 2 mg. of preparation NS7 (Table V).

DISCUSSION

It is clear that the bactericidal agent described in the preceding paper is effective *in vivo* as well as *in vitro* and protects mice against infection with virulent pneumococci. In fact, the protective action is observed not only when the agent is injected simultaneously with the infective dose, but also when it is administered several hours later.

Protection has been obtained against cultures of the five different types of pneumococci (I, II, III, V, VIII) which have been used; these cultures were of maximum virulence since 0.000,000,01 cc. was invariably fatal to mice within 72 hours. It is permissible to assume, therefore, that the agent will be found effective against experimental infection of mice with pneumococci of other types.

It is of special interest that the amount of bactericidal agent required to protect mice against a given amount of virulent culture is approximately the same irrespective of the type of pneumococcus used as infective agent. It is likely, therefore, that the action of the agent is directed against a structure or a function which is qualitatively and quantitatively similar in all pneumococci. The same conclusion had been derived from a study of

TABLE V
Effect of the Bactericidal Agent upon Experimental Infection of Mice with Klebsiella pneumoniae

Infective dose of <i>Klebsiella pneumoniae</i> type B	Treatment	Number of mice	Result			
cc.	mg.					
0.000,1	2	4	D 17	D 17	D 17	D 17
0.000,01	2	4	D 17	D 17	D 17	D 72
0.000,001	2	4	D 17	D 17	D 17	D 48
0.000,000,1	0	2	D 48	D 72		
0.000,000,01	0	2	D 72	D 72		

the action of the agent on pneumococci *in vitro*. As will be shown in a later publication, experiments carried out in collaboration with Dr. R. C. Lancefield have demonstrated that the agent also exerts a protective effect on experimental infection of mice with hemolytic streptococci of group A and C; on the contrary, it does not protect mice against *Klebsiella pneumoniae*, a Gram-negative organism, even when very small infective doses are used. This, again, is in agreement with the results of experiments *in vitro* in which a bactericidal effect was recognized against all the Gram-positive microorganisms so far tested, whereas the Gram-negative bacilli remained unaffected. There is little doubt, therefore, that the protective effect *in vivo* depends upon the same mechanism by which the bactericidal agent causes the death of the Gram-positive cells *in vitro*. It is interesting to contrast this direct bactericidal effect with the mechanism of the protection induced by a bacterial enzyme that hydrolyzes the capsular polysaccharide

of Type III Pneumococcus (2, 3). As described in earlier studies, this polysaccharidase does not in any way affect the viability of pneumococci; by decomposing the capsular substance of Type III organisms, however, it renders these bacterial cells susceptible to destruction by phagocytosis. The polysaccharidase does not attack the specific polysaccharides of other types of pneumococci, and consequently it protects only against infection with Type III organisms. On the contrary, the bactericidal agent considered in the present paper inhibits the growth of all Gram-positive organisms so far tested, and exerts on them a direct bactericidal action *in vitro* and *in vivo*.

SUMMARY

In the first paper of this series, a description was given of a cell-free extract, obtained from autolysates of a particular strain of a soil bacillus, which selectively inhibits the growth of all the Gram-positive microorganisms so far tested, and exerts on them a bactericidal effect *in vitro*.

In the present study it is shown that the same agent protects white mice against infection with large numbers of virulent pneumococci. It also exerts a curative effect when administered to mice several hours after injection of the infecting organisms.

The degree of protection afforded, and the minimal effective dose of bactericidal agent, are approximately the same for all virulent pneumococci, irrespective of type specificity.

The bactericidal agent is entirely ineffective against infection with virulent Friedländer bacilli (type B). This agrees with the fact that the agent does not affect Gram-negative bacilli *in vitro*.

The protective action exerted by the bactericidal agent against experimental pneumococcus infection depends upon the same mechanism which determines its bactericidal effect *in vitro*.

See Addendum to Paper I.

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AMOUNT AND DURATION OF IMMUNITY INDUCED BY INTRADERMAL INOCULATION OF CULTURED VACCINE VIRUS

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Jennerian prophylaxis in man by means of intradermal inoculation of cultured vaccine virus was described (1) in 1935. Since then this type of prophylaxis against smallpox has been used by us, by many private physicians, and by physicians in a number of clinics. For the last 3 years the virus employed has been from generations 50 to 170 of the "second revived" strain of cultured vaccine virus, dried from the frozen state in the presence of gum acacia and sealed *in vacuo* (1, 2). This strain (3) was originally derived from calf lymph vaccine virus supplied by the New York City Board of Health in 1931 and has been propagated since then by serial transfers in a medium consisting of Tyrode's solution and minced chick embryo tissue. Intradermal inoculation of the virus in rabbits reveals that it maintains a uniform potency during continued cultivation. Moreover, the lesions produced in rabbits are less severe than are those caused by other strains of vaccine virus.

When inoculated intradermally in susceptible persons, cultured vaccine virus produces a high percentage of positive reactions. The percentages reported by those to whom the virus has been dispensed have ranged from 80 to 100; it is probable that an average of over 90 per cent has been obtained in a minimum of 6000 to 7000 intradermal vaccinations. In our experience with more than 200 primary vaccinations the incidence of "takes" has been 100 per cent. Typical positive reactions appear as small red papules on the 4th to 9th day after inoculation. Erythema and induration increase until the lesions are 2 to 4 cm. in diameter at their height 4 to 6 days later. Erythema disappears rapidly although induration may persist 4 or 6 weeks. If

the inoculation is made properly, no vesicle forms to leave a scar. Those who have observed or experienced reactions produced in this way have been pleased by the lack of accompanying constitutional symptoms and the absence of open sores.

Knowledge of the amount and duration of immunity to smallpox induced by the intradermal injection of cultured vaccine virus is of cardinal importance. However, no opportunity of observing the incidence of smallpox in a group of individuals vaccinated in this manner has arisen. Nevertheless, from experience it is known that the efficacy of any type of vaccination against smallpox can be tested by revaccination with a potent calf lymph vaccine virus. In spite of this fact, reports of the effect of primary vaccination with cultured vaccine virus on subsequent revaccination with calf lymph virus have been few. In 1935 (1) we described the results obtained in the revaccination of 7 persons who had been successfully vaccinated with cultured virus 13 days to 7 months previously. Of these, 6 were immune to New York City calf lymph virus, while 1, after an interval of 7 months, responded with an accelerated take. In 1937 (4) we conducted revaccinations on a small group of children who had been vaccinated with cultured vaccine virus 1 month to 2½ years previously. In 6 of 14 such children the response to calf lymph virus was that of an accelerated take, *i.e.*, vesicles formed and the reactions were not at their maximum until the 5th or 6th day.

During the last year and a half we have studied a large group of children in order to obtain more complete information concerning the amount and duration of immunity produced by cultured vaccine virus against the New York City calf lymph strain of virus. The results of the study will be reported at this time. In addition, information regarding the immunity produced by cultured virus against other strains of calf lymph vaccine virus, as well as a consideration of the effect that differences in the manner of performing primary inoculations with cultured virus have on subsequent immunity, will be presented.

Methods

At the Rockefeller Hospital there is little opportunity of performing primary vaccinations, in consequence of which it has not been possible in this clinic for us to observe the response of a large number of children to revaccination. However,

at the Children's Prophylactic Clinic of the New York Hospital, cultured vaccine virus supplied by us has been administered intradermally for several years. The facilities and records of this clinic were made available to us through the courtesy of Dr. Samuel Levine and Dr. Parker Dooley.

Children were selected in whom a positive primary vaccination with cultured virus had been observed and recorded and in whom no further prophylaxis against smallpox had been carried out. Each child was revaccinated with New York City Board of Health calf lymph vaccine virus applied to a linear scratch $\frac{1}{8}$ inch in length. In addition various groups received on the opposite limb commercial calf lymph virus A or B applied to a linear scratch. All virus used was received fresh each week from the place of preparation and was stored at 0°C. before use. A single observation on the 5th day after revaccination has, as a rule, been all that could be made; the few that could not be seen on the 5th day were seen between the 4th to 7th days.

The time at which a reaction to vaccine virus is at its maximum and not the size of the lesion is considered to be the correct index of susceptibility (5). Reactions are usually classified as no reaction, immune reaction, accelerated take, primary take. Due to the impossibility of making frequent observations on the revaccinated children only two types of reaction are recorded, namely, immune reactions and accelerated takes. Immune reactions are those which showed on the 5th day only a small papule or some evidence that a mild response to inoculation had been present. The children who showed at this time no evidence that the virus had been effectively introduced into the skin were excluded from consideration. Thus, a few rapid immune reactions may have been missed, but the number was not great enough to affect significantly the results of the study. Accelerated reactions comprise those which on the 5th day showed the presence of a vesicle surrounded by a zone of erythema. The use of vesicle formation as one of the criteria for classification has insured the inclusion in the group of accelerated takes of even the mildest of this kind of reaction, *viz.*, one which heals without the formation of an enduring scar. It is possible that some of the reactions may have been in an early stage when the results were recorded and that the time at which the maximum sizes were reached approached closely the time at which primary takes would have been at their height. However, from observation of some children later than the 5th day after inoculation and from the size of resultant scars which we have seen, we believe that most of the accelerated reactions were correctly classified.

Results of Revaccination with New York City Calf Lymph Vaccine Virus of Children Who Had Received One Successful Intradermal Inoculation of Cultured Vaccine Virus

331 children who had received one inoculation of cultured virus resulting in a primary take 1 month to 3 years and 9 months previously were revaccinated with New York City calf lymph virus (Table I).

Of these, 82 or 25 per cent responded with immune reactions, while 249 or 75 per cent showed accelerated takes. Most of the accelerated reactions were mild in character, presenting a small vesicle surrounded by a zone of erythema 0.5 to 1 cm. in width. Frequently on the 5th day the contents of the vesicles were drying or inquiry revealed that the lesions had been larger or as large on the preceding day. Children responding in this manner did not present the usual symptoms and signs that as a rule accompany primary vaccination with calf lymph. There were others, however, in whom the lesions presented no signs

TABLE I

Results of Revaccination with New York City Vaccine Virus of Children Who Had Received One Successful Intradermal Inoculation of Cultured Virus

Number of children revaccinated	Time between primary and secondary vaccinations	Immune reactions		Accelerated takes	
		Number	Per cent	Number	Per cent
39	1-6 mos.	13	33	26	67
76	6 mos.-1 yr.	25	33	51	67
185	1-2 yrs.	37	20	148	80
31	2+ yrs.	7	23	24	77
Total 331		82	25	249	75

Distribution of age at time of primary vaccination similar for all groups.

of regression on the 5th day and who experienced later fever and lymph gland enlargement accompanying the presence of a central pustule in a zone of erythema and induration of considerable extent. Nevertheless, healing of these lesions was rapid and the scars which resulted were small and superficial.

Analysis of the data obtained in this group of 331 children revealed that the proportion of immune individuals was fairly constant and bore no relation to the interval which had elapsed between the primary vaccination with cultured virus and revaccination with calf lymph (Table I). It is true that the percentage of accelerated reactions was slightly higher in the children revaccinated after 1 year and that the more severe reactions were observed in this group, but the figures obtained give little indication that susceptibility to calf lymph virus increased with the lapse of time within the limits of the observations.

It is known that infants shortly after birth (6) are somewhat resistant to infection with vaccine virus. Furthermore, it has been demonstrated (6) that such infants after a successful vaccination rapidly lose their immunity, many being fully susceptible a year later. The results obtained in our group of 331 children, the majority of whom were first vaccinated between the ages of 6 months and 1 year, indicate that the differences in the age at which the primary vaccinations were performed had no influence on the proportion of children

TABLE II

Results of Dermal Revaccination Made with New York City Vaccine Virus to Ascertain the Duration of Immunity Produced by One Successful Intradermal Inoculation of Cultured Virus in Relation to the Age of Children at Time of Primary Vaccination

Number of children revaccinated	Age when first vaccinated	Immune reactions		Accelerated takes	
		Number	Per cent	Number	Per cent
69	6-9 mos.	15	22	54	78
98	9 mos.-1 yr.	29	30	69	70
55	1-2 yrs.	14	25	41	75
35	2-3 yrs.	7	20	28	80
53	3-5 yrs.	11	21	42	79
21	5+ yrs.	6	29	15	71
Total 331		82	25	249	75

Distribution of interval of time between primary and secondary vaccinations similar for all groups.

who retained complete immunity during the period of observation (Table II).

Results of Revaccination with Commercial Strains of Vaccine Virus of Children Who Had Received One Successful Intradermal Inoculation of Cultured Vaccine Virus

Reports in the literature concerning the duration of immunity in children to vaccine virus are conflicting. Moreover, in attempting to evaluate the results of different workers, one is confused by a lack of uniformity in classification or description of the type of reaction produced by revaccination and by the fact that the relative potency

of the viruses used for the primary vaccinations and revaccinations was either not known or not stated. A mildly acting virus does not always fully protect for a great length of time against a virulent strain, and results obtained by revaccination with a mild strain may not parallel those secured by revaccination with a potent virus. The New York City vaccine virus is a strain of high uniform potency. Consequently, it seemed of interest to compare the results obtained by

TABLE III

Results of Dermal Revaccination with Commercial Strains of Vaccine Virus of Children Who Had Received One Successful Intradermal Inoculation of Cultured Virus

Number of children revaccinated	Revaccinated with New York City virus		Revaccinated with commercial virus A		Revaccinated with commercial virus B	
	Per cent immune reactions	Per cent accelerated takes	Per cent immune reactions	Per cent accelerated takes	Per cent immune reactions	Per cent accelerated takes
78	22	78	72	28		
82	35	65			55	45

Distribution of age at time of primary vaccination and interval of time between primary and secondary vaccinations similar in both groups.

means of its use in the revaccination of children with those secured by revaccination with other strains of calf lymph vaccine virus.

Two commercial preparations of calf lymph virus, A and B, were chosen because they are products widely used in the United States. 78 of the 331 children who were revaccinated with New York City calf lymph received at the same time an inoculation with commercial lymph A; a second group of 82 children received in addition to New York City virus an inoculation of commercial calf lymph B. Of the 78 children, 17 or 22 per cent responded with immune reactions to New York City virus, while 56 or 72 per cent responded with immune reactions to calf lymph A; of the 82 children, 29 or 35 per cent were immune to New York City virus, while 45 or 55 per cent responded in that manner to calf lymph B. These figures, recorded in Table III, show discrepancies that may result from the use of different strains of virus.

*Effect of Differences in Primary Inoculation of Cultured Vaccine Virus
on Subsequent Revaccination with New York City Board of
Health Calf Lymph*

At this point it seemed of value to learn whether the administration of large doses of the mildly acting cultured virus or the production of 2 intradermal lesions at the same time would influence the resultant immunity. Accordingly, a group of children at the New York Hospital Clinic were given 2 intradermal inoculations, one in each arm or thigh, of cultured vaccine virus. There were no untoward results. The simultaneous evolution of 2 intradermal vaccinal lesions produced by cultured vaccine virus apparently caused the children no more inconvenience than that evoked by a single reaction. From this group of children, 66, whose records showed that they had had 2 successful simultaneous primary vaccinations, were revaccinated dermally with New York City calf lymph 2 to 6 months later. Of the 66 children, 18 or 27 per cent responded with immune reactions, while 48 or 73 per cent showed accelerated takes. Comparison of these figures (Table IV) with those obtained in the group of 331 (Table I) who received only a single injection of cultured virus for primary vaccination shows that the introduction of a double amount of this virus and the production of 2 primary lesions instead of one did not alter the percentage of children who retained for 6 months complete immunity to the New York City calf lymph.

As stated previously, the virus which has been used during the last 3 years was obtained from the 50th to the 170th culture generations of the "second revived" strain. This virus was selected for human inoculation because it produced mild reactions and maintained a constant potency for man and rabbit. However, from previous experience (2) with the original strain we had noted that the infectivity of the virus diminished on repeated passage in culture and that a change in the character of the lesions produced by it in rabbits also occurred during serial transfer of the virus in the medium used. It occurred to us that a gradual change might have taken place in the "second revived" strain, less marked than that noted in the original one but still great enough to influence the amount of protection produced against a highly potent strain of vaccine virus or against

smallpox. Therefore, it seemed important to determine whether continued cultivation of the "second revived" strain had resulted in a loss of some of its antigenicity essential for the development of a lasting immunity. In order to make this determination, cultured virus from the 20th to the 30th generations of the "second revived" strain was prepared for human inoculation and tested in rabbits and in man.

Intradermal inoculation of the virus in rabbits revealed that the infectivity or titer was essentially the same as that of generation 50 to 170, but the lesions produced by the early generations were more edematous and more hemorrhagic and necrotic than were those

TABLE IV

Results Obtained by Dermal Revaccination with New York City Vaccine Virus of Children Who Had Been Primarily Vaccinated Intradermally in Several Different Ways with Cultured Virus

Type of primary inoculation	Number of children revaccinated	Number of immune reactions	Per cent of immune reactions	Number of accelerated takes	Per cent of accelerated takes
Single inoculation with virus from 50-170th culture generation	331	82	25	249	75
Double inoculation with virus from 50-170th culture generation	66	18	27	48	73
Single inoculation with virus from 20-30th culture generation	54	33	61	21	39

produced by later generations of the active agent. Each of 7 volunteers was inoculated intradermally with 0.1 cc. of a 1:10 dilution of the virus. The lesions produced by this material were larger and more severe than those caused by virus from later generations; however, the reactions were not severe enough to cause anxiety regarding the use of the material. Consequently, a study of immunity produced by it was carried out in a group of children at the New York Hospital Prophylactic Clinic. 54 children were inoculated intradermally with cultured virus from generations 20 to 30; 2 to 6 months later they were reinoculated dermally with the New York City calf lymph virus. Of the 54 children, 21 or 39 per cent responded with accelerated takes, while 33 or 61 per cent showed immune reactions (Table IV). The accelerated takes were mild and healed quickly leaving only small superficial scars. These results are significantly different from those

obtained in children primarily vaccinated with virus from culture generations 50 to 170.

DISCUSSION

Vaccination against smallpox by means of dermal application of potent calf lymph vaccine virus is efficacious. Nevertheless, considerable inconvenience and, at times, danger are associated with this type of vaccination which always leaves an ugly scar. In view of these facts, many people in the United States have never been vaccinated. To overcome opposition to vaccination certain health officials minimize the inconvenience and speak of the scar as a "badge of health." Thus, they leave the impression that a person with a scar is protected against smallpox and is not in need of revaccinations at regular intervals. As a matter of fact, all that a scar indicates is that an individual has been vaccinated; it does not show that the person is immune to smallpox. That can be determined only by the results of revaccination with a potent calf lymph virus. Moreover, the longer a person has gone since a primary vaccination, the more likely is he to have lost protection and the greater is his need of revaccination. Some individuals lose immunity more rapidly than do others; this appears to be particularly true of young children and infants. Therefore, revaccinations should be made at regular intervals; in the presence of smallpox epidemics revaccinations should be made regardless of when primary vaccinations or revaccinations were performed.

With the idea that vaccination against smallpox can be made a safer procedure, that mutilation is not an essential feature of the procedure, and that a scar gives the individual as well as the health officer a false sense of security, we undertook a number of years ago to prepare a vaccine virus that could be used in a manner less objectionable than that now employed with calf lymph virus. From the results obtained by us in the use of cultured vaccine virus for Jennerian prophylaxis in man we have become convinced that the ideas which prompted the work are entirely sound.

Continued cultivation of vaccine virus in the medium used by us has brought about a qualitative change in the active agent which makes it possible to introduce considerable amounts of the material intradermally without danger and inconvenience to patients. It has been found, however, that the amount of immunity produced by the

cultured virus, as tested by means of a highly potent calf lymph vaccine virus, would probably not be considered sufficient for complete protection against smallpox. On the other hand, when commercial vaccines widely used in the United States are employed for testing the immunity induced by the cultured virus, the results might be considered satisfactory. At present we are suggesting that primary vaccinations be made intradermally with our cultured virus and that revaccinations be made dermally six months to a year later by means of a potent calf lymph virus. In this way vaccinated individuals will not become sick and will not be subjected to the dangers associated with primary vaccinations with calf lymph virus, but will obtain a solid and lasting immunity to smallpox. It is possible and highly probable that a cultured virus can be developed which will be suitable for intradermal use and will not require prompt dermal revaccinations with a potent calf lymph virus to produce an enduring immunity. These are matters for future investigation.

CONCLUSIONS

Continued cultivation of vaccine virus in a medium consisting of minced chick embryo tissue and Tyrode's solution has resulted in a virus qualitatively changed to such an extent that considerable amounts of it can be injected intradermally into human beings without danger or inconvenience.

Individuals who are vaccinated intradermally with the cultured virus should be revaccinated dermally six months to a year later with a potent calf lymph virus in order to obtain a satisfactory immunity to smallpox without being subjected to the dangers and inconvenience associated with primary vaccinations with calf lymph virus.

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A SOLUBLE ANTIGEN OF LYMPHOCYTIC CHORIOMENINGITIS

I. SEPARATION OF SOLUBLE ANTIGEN FROM VIRUS

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Soluble antigens separable from the etiological agents have been found in a number of virus infections, *e.g.*, bacteriophagy (1), yellow fever (2), vaccinia (3), psittacosis (4), influenza (5), and myxomatosis (6). On the other hand, in certain virus diseases, *e.g.*, papillomatosis of rabbits (7) and tobacco mosaic of plants (8), all the *in vitro* serological phenomena appear to be the result of reactions between immune sera and the viruses. The occurrence of a complement-fixing substance in extracts prepared from brains of guinea pigs and mice infected with the virus of lymphocytic choriomeningitis has been reported by Howitt (9). However, she did not mention any attempts to determine whether complement fixation was due to the presence of virus or to a soluble material separable from the virus. Separation of a complement-fixing antigen from the virus has been reported by us in a preliminary publication (10). The purpose of this paper is to present in detail the evidence for the existence of a specific soluble antigen associated with the virus of lymphocytic choriomeningitis, and to record its distribution in organs of infected animals.

For experiments of this type, it is essential to have materials rich both in virus and in complement-fixing antigen. The virus of lymphocytic choriomeningitis is not only neurotropic, but is also highly viscerotropic, since it is found in relatively large amounts in organs other than the brain and occurs even in the blood of acutely ill animals (11, 12). Preliminary experiments indicated that spleens from infected guinea pigs were suitable for our needs; hence, material from this source has been extensively used.

Materials and Methods

Source of Virus and Complement-Fixing Antigen.—Guinea pigs were inoculated intracerebrally with 0.1 cc. and subcutaneously and intraperitoneally, respectively, with 1.0 cc. of a 10 per cent suspension of fresh brain from guinea pigs infected with one of four strains of the virus of lymphocytic choriomeningitis maintained in Dr. Rivers'

laboratory (W.E., F.A., R.E.S., and W.W.S.). Animals were sacrificed at the height of the disease, that is, on the 5th or 6th day when infected with the W.E. strain, and on the 9th or 10th day when infected with the W.W.S. strain; organs were removed aseptically, weighed, ground, and made up to a 10 per cent suspension with a physiological saline solution which usually contained 2 per cent normal inactivated guinea pig serum. Mice were inoculated with the W.E. strain of virus by the intracerebral and intraperitoneal routes and sacrificed on the 7th day.

Titration of Infectivity of Virus.—The infective titer of virus preparations was determined in mice by intracerebral inoculations of 0.025 cc. of tenfold dilutions of material. Three mice as a rule received each dilution and survivors were discarded 21 days after inoculation. In experiments in which the virus was concentrated or washed, the various materials to be tested were injected into animals at about the same time. Thus, for control a portion of the original tissue suspension was kept at room temperature, usually for 6 to 8 hours, until the final preparation of virus was obtained.

Preparation of Complement-Fixing Antigen.—Coarse particles were removed by centrifugation at slow speed from 10 per cent suspensions of infected organs which were prepared as described above. The turbid supernatant fluid was then run in the air-driven, concentration centrifuge of Bauer and Pickels (13). The clear, claret colored ultrasupernatant fluid, carefully separated from the floating material and the tan sediment by means of a capillary pipette, was filtered through a Seitz pad which had been previously prepared with saline solution containing 10 per cent inactivated normal guinea pig serum.

Antisera.—Antisera used most extensively were obtained from guinea pigs hyperimmunized in the following manner with the virus of lymphocytic choriomeningitis. Animals which had recovered from infection with the W.W.S. strain of virus, which has a low pathogenicity for guinea pigs, were inoculated intraperitoneally with 2 to 3 cc. of a 10 per cent suspension of brain tissue from guinea pigs infected with the highly virulent W.E. strain. Animals which survived this reinoculation for 10 days were again injected with a similar amount of active material; 10 days later they were bled from the heart under light anesthesia and their pooled serum constituted the hyperimmune serum used. Simple immune serum was obtained from guinea pigs which had recovered from infection with the W.W.S. strain of virus.

Complement-Fixation Reaction.—Materials employed in the hemolytic system were as follows: anti-sheep cell hemolysin, prepared in rabbits, was diluted so that 0.2 cc. contained 2 units (generally a 1:800 dilution). Guinea pig serum for complement was obtained in large amounts, desiccated *in vacuo* from the frozen state (14), and stored at 0°C. At the time of the test, dried serum was brought back to its original volume by the addition of distilled water. Two units of hemolysin and 0.5 cc. of a 5 per cent suspension of washed sheep erythrocytes were added to varying dilutions of resuspended serum in order to determine the titer of complement. Estimation of hemolysis was made after the tubes had been incubated in a water bath at 37°C. for 30 minutes. The titer of the resuspended complement was usually such that 0.03 cc. represented 2 units.

The test was performed in the following manner: 0.2 cc. of a solution of antigen, 0.2 cc. of inactivated hyperimmune serum, and 0.2 cc. of diluted complement (2 units) were mixed and stored overnight at 2°C. 0.7 cc. of a suspension of sensitized erythrocytes were added to each tube the following morning; this suspension was prepared by mixing an appropriately diluted solution of hemolysin with a 5 per cent suspension of sheep

erythrocytes, washed 3 times, in a proportion of 2:5. The tubes were kept in a water bath at 37°C. for 30 minutes, after which time the degree of hemolysis was estimated. The activity of the hemolytic system was such that complete lysis of appropriate controls usually occurred in 10 to 15 minutes. Suitable anticomplementary and hemolytic control systems were included in each test. Dilutions of antigen and serum were calculated on the basis of material in the 0.2 cc. used in the test; the titer was taken as the highest dilution which bound complement completely.

EXPERIMENTAL

Concentration of Virus

The particles of the virus of lymphocytic choriomeningitis have been reported to possess diameters not greater than 100 to 150 $m\mu$ (15). We have found that this medium sized virus was not appreciably sedimented by a technique adequate for concentration of the virus of vaccinia (16), namely, that of centrifugation in narrow flat tubes in a Swedish angle centrifuge at 3500 R.P.M. for 1 hour. On the other hand, we have been able to concentrate the virus by high speed centrifugation (13), 30,000 R.P.M. for 20 minutes, or 20,000 R.P.M. for 30 minutes being sufficient to sediment the virus.

Early attempts to wash the ultrasediment with saline solution were unsuccessful, inasmuch as repeatedly washed material possessed little virus activity. It was found, however, that tissues heavily laden with virus rapidly diminished in infectivity when suspended in saline solution for a few hours at room temperature. In view of the toxicity of sodium chloride solution for the virus of herpes simplex (17), diluents other than saline solution were tried, e.g., dilute sodium phosphate-citric acid buffer solution at pH 7.2, dilute potassium phosphate-citric acid buffer solution at pH 7.6, and bacteriological broth. Although none of these diluents was satisfactory, the least inactivation of virus occurred when broth was used. Since the addition of normal serum to saline suspensions of the virus of yellow fever (18) renders this infectious agent more stable on storage, a similar procedure was followed in our experiments. Physiological saline solution containing 2 per cent normal inactivated guinea pig serum proved to be a satisfactory menstruum for our purpose, since with it infectious sediments could be repeatedly washed, as shown by the following work, without appreciable loss of activity.

The ultrasediment from 50 cc. of a 10 per cent suspension of infected splenic tissue which had previously been run in the horizontal machine usually amounted to 0.5 or 0.6 cc. When this sediment was resuspended in 10 to 20 cc. of diluent and then partially cleared by horizontal centrifugation at 2500 R.P.M. for 30 minutes, a considerable amount of sediment (± 0.3 cc.) resulted, leaving a moderately opaque supernatant fluid.

The removal of this large amount of sediment by horizontal centrifugation did not materially reduce the infectivity of the supernatant fluid. A method of differential centrifugation was then introduced into the technique for washing the virus; for example, the sediment resulting from high speed centrifugation was resuspended and run at 10,000 R.P.M. for from 6 to 10 minutes in the ultracentrifuge. The slow ultracentrifugation, like the horizontal run, gave a considerable amount of sediment which only slightly reduced the infectivity of the supernatant fluid. Hence, the supernatant suspension from the slow speed centrifugation was saved while the sediment was discarded. Differential centrifugation of this type, with alternate fast and slow runs, always resulted in some loss of virus when the sediment was washed 3 or 4 times. In typical experiments about 0.15 cc. of washed sediment was ultimately obtained from 10 infected spleens. This material, resuspended in 20 cc. of serum-saline solution and run at 10,000 R.P.M. for 6 minutes, gave a faintly opalescent supernatant fluid which was infective in a dilution of 10^{-5} or 10^{-6} . Original splenic suspensions were infective at dilutions of 10^{-7} or occasionally 10^{-8} . Suspensions of washed virus did not form an appreciable amount of sediment when stored for several weeks at 3°C ., that is they were stable. Moreover, although the infectivity gradually decreased on storage, one specimen of washed virus with an original titer of 10^{-7} still killed animals when diluted to 10^{-3} after storage for 4 weeks at 3°C .

The appreciable loss in infectivity, which occurred when the above method of differential centrifugation was employed, led us to modify the technique. Ultrasediment was washed 3 or 4 times but centrifugation at 10,000 R.P.M. was omitted until after the ultrasediment had been resuspended for the last time. Suspensions of washed virus obtained by this method had an appearance similar to that of the previous preparations but were as infectious as the original suspensions of infected spleen (Table I).

Results of attempts to demonstrate an agglutination phenomenon with suspensions of washed virus were not sufficiently clear cut to be convincing. Examination of suspensions under dark field illumination revealed many tiny particles of uniform size which appeared smaller than elementary bodies of vaccinia viewed under similar conditions. The explanation of the failure to obtain definite agglutination may depend upon the fact that insufficient virus was present. Thus, Parker and Rivers (19) observed that the concentration of elementary bodies of vaccinia required for visible agglutination was log 8.29 particles per cc. On the basis of Merrill's studies (20), the number of particles per cc. of the virus of lymphocytic choriomeningitis necessary for a visible agglutination reaction would be greater than the number of Paschen bodies. It is at once apparent that the concentration of active virus of lymphocytic choriomeningitis in the final preparations was even less than that required for an agglutination reaction with elementary bodies of vaccinia. Furthermore, only a portion of the particles visible in the dark field could have been bodies of virus, since

preparations made by the same technique from spleens of normal guinea pigs contained at least half as many small, uniform particles as did the preparations made from infected spleens.

Separation of Complement-Fixing Antigen from Virus by Means of Ultracentrifugation

In the preceding experiments it was found that virus can be readily sedimented from suspensions of infected spleen by ultracentrifugation at 30,000 R.P.M. for 20 minutes. The supernatant fluid, freed of virus, fixed complement in the presence of immune serum as well as the uncentrifuged material. On the other hand, particles of virus which had been washed several times were capable of binding complement only to a slight extent. The following experiment is typical of five which gave similar results.

Spleens were removed aseptically from 10 guinea pigs 6 days after infection with the W.E. strain of virus. The organs were weighed, ground in a mortar, and made up to a 10 per cent suspension with buffered saline solution, pH 7.2, containing 2 per cent inactivated normal guinea pig serum. 50 cc. of a supernatant suspension were obtained after centrifugation at 2500 R.P.M. for 20 minutes. 15 cc. of this were set aside for determination of infectivity and complement-fixing titer, both before and after Seitz filtration. The remaining 35 cc. were placed in lusteroid tubes¹ and run at 30,000 R.P.M. for 20 minutes. Floating material was removed with a cotton swab and the clear supernatant fluid was taken up with a capillary pipette; residual particulate material was removed from the supernatant fluid by another run at the same speed; then the fluid was filtered through a Seitz pad and saved for titrations. The sediment from the original ultracentrifugation which contained the virus was thoroughly dispersed in 15 cc. of serum-saline solution and again sedimented. In this manner, the virus was washed 3 times, and then taken up in a volume of 15 cc.; the first 2 wash waters were saved. The virus suspension was next centrifuged at 10,000 R.P.M. for 6 minutes; the faintly opalescent supernatant fluid was considered as the final suspension of virus; the sediment was discarded.

The results of this experiment are summarized in Table I. The suspension of infected spleens after the first horizontal centrifugation gave complete fixation of complement when diluted 1:32, but was anticomplementary in low dilutions; when diluted 10^{-8} it was infectious for all mice inoculated, and killed 1 of 3 mice which received the next higher dilution. A marked reduction in infectivity and a slight but detectable diminution in amount of complement-fixing antigen occurred when the crude suspension was filtered through a Seitz pad. Ultracentrifugation of the crude suspension, followed by Seitz filtration of the supernatant fluid, resulted

¹ Manufactured by the Lusteroid Container Corporation, South Orange, New Jersey.

in a complete loss of infectivity for mice. However, the complement-fixing antigen was still present in the same amount as in the previous filtrate. Decreasing amounts of antigen were present in successive lots of fluid which were used to wash the sedimented virus. The final preparation of washed

TABLE I
Separation of Soluble Antigen from Virus in Infected Splenic Tissue by Centrifugation and Filtration

Material	Infective titer	Approximate dilution of original splenic tissue	Dilution of material					
			1:1	1:2	1:4	1:8	1:16	1:32
Horizontal supernatant unfiltered	10^{-6}	10^{-1}	Anticomplementary			++++	++++	++++
Horizontal supernatant Seitz filtrate	10^{-3}	10^{-1}	++++	++++	++++	++++	+++	—
Ultr supernatant Seitz filtrate	Negative	10^{-1}	++++	++++	++++	++++	+++	—
1st wash water Seitz filtrate	Not done	10^{-2}	++++	++++	+++			
2nd wash water Seitz filtrate	" "	10^{-2}	+++	—	—			
Final virus suspension washed 3 × (in $\frac{1}{2}$ original volume)	10^{-7}	10^{-6}	+++	+++	±	—	—	—

Guinea pigs were infected with W.E. strain of L-C-M virus.

Original suspending medium and "wash water" consisted of buffered saline solution containing 2 per cent heated normal guinea pig serum.

Preliminary centrifugation was done in horizontal machine at 2500 R.P.M. for 20 minutes; ultracentrifugation at 30,000 R.P.M. for 20 minutes.

Degree of complement fixation is indicated in the usual manner.

virus was as infectious as the original suspension of spleen; it killed all 3 mice which were inoculated with a 10^{-6} dilution and 2 of the 3 mice which received a 10^{-7} dilution. In contrast to the high infective titer the final virus preparation, in a dilution of 1:2, reacted incompletely with complement. Final suspensions of virus were not anticomplementary even in the undiluted state, provided that aggregated particles were removed by a final centrifugation at 10,000 R.P.M. When this procedure was omitted, the

lower dilutions of virus suspension were anticomplementary and the slight fixation displayed by the virus was missed.

The ease with which the complement-fixing antigen can be separated from the infective agent indicates that lymphocytic choriomeningitis belongs to the group of virus infections in which is found a specific soluble antigen separable from the virus. Moreover, as in vaccinia (21) and psittacosis (22), the washed particles of the virus of lymphocytic choriomeningitis are capable of fixing complement to a certain degree.

Distribution of Soluble Antigen in Organs of Infected Animals

Antigen prepared according to the routine method from infected spleens of guinea pigs usually gave complete fixation of complement when 0.2 cc. of a dilution of 1:16 were mixed with hyperimmune serum. However, soluble antigen was occasionally not demonstrable in such preparations in a dilution greater than 1:4; on the other hand, certain lots fixed complement in a dilution of 1:32. The distribution of complement-fixing antigen in a number of tissues and in the blood of infected guinea pigs was next investigated. Antigenic material, prepared according to routine from lungs showing pneumonic consolidation, completely fixed complement in the undiluted state. Moreover, in several experiments, undiluted extract obtained from livers was shown also to fix complement partially. On only two occasions did extracts of infected brains give even partial fixation. Soluble antigen was not present in detectable amounts in extracts of kidney nor was it demonstrable in the serum, although spleens of the same animals contained antigen in large amounts. Furthermore, the lots of pooled serum which were tested for the presence of antigen were infective in dilutions of 10^{-5} and 10^{-6} ; the serum was used as antigen after being heated for 15 minutes at 56°C . in order to inactivate complement which might have been present.

The presence of soluble antigen was also demonstrated in extracts prepared from organs of infected mice. The amounts of antigen obtained from spleen and liver were comparable to those which occurred in extracts of these organs of infected guinea pigs. In neither of two experiments was antigen found in demonstrable quantity in preparations from brains of infected mice.

Finally, complement-fixing antigen was demonstrated in an extract of consolidated lung obtained from a monkey dead of lymphocytic choriomeningitis.

One experiment was made with materials from a monkey (*Macacus rhesus*) infected by the subcutaneous route with 10^5 mouse doses of virus of the W.E. strain. The

animal developed fever 48 hours after injection and died on the 12th day. Blood taken on the 11th day was infectious for guinea pigs but contained neither soluble antigen nor complement-fixing antibody in demonstrable amounts. An extensive hemorrhagic pneumonia was found at autopsy performed immediately after death. Consolidated portions of lung were ground and made into a 15 per cent suspension (wet weight). The infective titer of the crude suspension of lung was found to be 10^{-6} . After preparation by the usual centrifugation technique the lung extract fixed complement when diluted 1:16. The spleen was not used because of the presence of tubercles.

TABLE II
*Distribution of Soluble Antigen in Material from Animals Infected
with Virus of Lymphocytic Choriomeningitis*

Species	Source of antigen	Dilution of antigen					
		1:1	1:2	1:4	1:8	1:16	1:32
Guinea pig	Spleen	++++	++++	++++	++++	++	—
	Lung*	++++	++—	—	—	—	—
	Liver	++	—	—	—	—	—
	Brain	++	—	—	—	—	—
	Kidney	—	—	—	—	—	—
	Serum	—	—	—	—	—	—
Mouse	Spleen	++++	++++	+++	±	—	—
	Liver	+++	—	—	—	—	—
	Brain	—	—	—	—	—	—
Monkey	Lung*	++++	++++	++++	++++	++++	—
	Serum	—	—	—	—	—	—

* Only areas showing pneumonic consolidation were employed.

Hyperimmune guinea pig serum was used in a dilution of 1:32.

Animals infected with W.E. strain of virus were sacrificed at the height of the disease. 10 per cent suspensions of fresh organs were subjected to ultracentrifugation and Seitz filtration, except in the case of the monkey lung (see text).

Degree of complement fixation is indicated in the usual manner.

A comparison of the distribution of complement-fixing antigen in material obtained from acutely ill animals is shown in Table II.

*Occurrence of Antigen in Guinea Pigs Infected with Different Strains
of the Virus*

Four strains of the virus of lymphocytic choriomeningitis maintained in this laboratory vary in their pathogenicity for the guinea pig. The W.E. strain, with which most of the present work is concerned, is almost invariably lethal for guinea pigs when inoculated either intracerebrally or

subcutaneously. The brain of a guinea pig moribund after inoculation of this strain is infectious in a dilution of 10^{-7} or 10^{-8} when passed intracerebrally to other guinea pigs. On the other hand, the W.W.S. strain rarely kills guinea pigs when administered even in large amounts by either of these routes. Moreover, it has a low pathogenicity for mice. Two other strains, F.A. and R.E.S., behave in a manner intermediate between the two just mentioned. In view of these facts, it was of interest to determine whether the presence of complement-fixing antigen could be demonstrated in preparations of spleens from animals infected with the 3 less virulent

TABLE III
Occurrence of Soluble Antigen in Spleens of Guinea Pigs Infected with Four Strains of Virus

Guinea pig serum	Strain of virus	Dilution of antigen					
		1:1	1:2	1:4	1:8	1:16	1:32
W.E. hyperimmune (dilution 1:32)	W.E.	++++	++++	++++	++++	+	-
	F.A.	++++	++++	-	-	-	-
	R.E.S.	++++	+++	-	-	-	-
	W.W.S.	++	-	-	-	-	-
W.W.S. immune (dilution 1:32)	W.E.	++++	++++	++++	++++	+++	-
	F.A.	+++++	++	-	-	-	-
	R.E.S.	++++	-	-	-	-	-
	W.W.S.	±	-	-	-	-	-

10 per cent suspensions of infected splenic tissue were subjected to ultracentrifugation and Seitz filtration.

Degree of complement fixation is indicated in the usual manner.

strains of virus. Furthermore, it was desirable to know whether the soluble antigen exhibited any evidence of strain specificity. Accordingly, splenic extracts were prepared in the usual manner from guinea pigs infected with each of the 4 strains of virus and tested against the serum of a guinea pig immune to the W.W.S. strain and against the pooled hyperimmune serum used throughout the experiments.

From the results shown in Table III, it can be seen that splenic extracts prepared from animals infected with each strain of virus contained soluble antigen. Moreover, no evidence of type specificity was encountered in the 4 strains. However, certain quantitative differences were noted in the preparations, *viz.*, the amount of antigen obtained from spleens of animals infected with the 4 strains corresponded directly to the virulence of each strain. This observation might be interpreted to mean that the soluble

antigen contributes to the virulence of the strain of virus. On the other hand, infection with the most virulent strain (W.E.) results in a higher concentration of demonstrable virus in tissues of animals than does infection with the milder strains. This greater amount of virus might easily account for the presence of more soluble antigen in tissues infected with the virulent strain, irrespective of the origin of the soluble substance, *i.e.*, virus particle or host cell.

Specificity of the Complement-Fixation Reaction

Extracts of normal organs of the guinea pig, mouse, and monkey, prepared according to the routine procedure, did not fix complement in the presence of lymphocytic choriomeningitis hyperimmune serum. Nor did serum from the normal guinea pig, mouse, or monkey react with organ extracts of the homologous species which contained the soluble antigen. Howitt (9) observed no cross-reaction when materials obtained from animals infected with the viruses of lymphocytic choriomeningitis, St. Louis encephalitis, and several strains of equine encephalomyelitis, were employed in complement-fixation tests. Similarly, we have found no evidence of cross-reaction in tests in which the soluble antigen of lymphocytic choriomeningitis was mixed with serum from rabbits hyperimmunized with the virus of vaccinia or of myxomatosis. Furthermore, no detectable reaction occurred when the antigen was used with human serum which contained antibodies against the soluble antigen of influenza.² Finally, solutions of antigen, which reacted specifically with antisera for vaccinia, myxomatosis, and influenza, respectively, gave no fixation of complement when added to serum from guinea pigs hyperimmunized with the virus of lymphocytic choriomeningitis. Results of experiments dealing with the specificity of the reaction are shown in Table IV.

Complement-fixing antigens from sources other than virus-infected tissues were also tested. No cross-reactions were observed by Eaton (23) between malarial antiserum and malarial antigen, respectively, and soluble substance of lymphocytic choriomeningitis and hyperimmune guinea pig serum which we supplied. Furthermore, human sera known to give positive Wassermann reactions³ did not react with the soluble antigen of lymphocytic choriomeningitis; this finding agrees with observations of Lépine, Mollaret, and Sautter (24). In addition, other human sera capable of fixing complement in the presence of gonococcal antigen³ gave no reaction with the antigen of lymphocytic choriomeningitis.

² Influenzal antigen and antiserum were provided by Dr. Frank L. Horsfall, Jr.

³ Known positive and negative Wassermann and gonococcal complement-fixing sera were provided by Dr. Archibald McNeil.

TABLE IV
Specificity of Soluble Antigen of *Lymphocytic Choriomeningitis*

Antiserum	Source of antigen	Dilution of antigen	Dilution of antiserum									
			1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Vaccinia hyperimmune (rabbit)	Vaccinia, dermal filtrate	1:25	++++	++++	++++	++++	++++	++++	++++	++++	+	-
	L-C-M, spleen	1:3						-	-	-	-	-
Myxoma hyperimmune (rabbit)	Myxoma, acute serum	1:25	++++	++++	++++	++++	++++	++++	++++	++++	++	-
	L-C-M, spleen	1:3						-	-	-	-	-
Influenza convalescent (human)	Influenza, mouse lung	1:10	++++	++++	++++	++++	++++	±				
	L-C-M, spleen	1:3	-	-	-	-	-	-				
L-C-M hyperimmune (guinea pig)	L-C-M, spleen	1:3						++++	++++	++++	++++	-
	Vaccinia, dermal filtrate	1:25						-	-	-	-	-
	Myxoma, acute serum	1:25						-	-	-	-	-
	Influenza, mouse lung	1:10						-	-	-	-	-

Extracts of tissue prepared from normal animals did not contain specific soluble antigen.
Degree of complement fixation is indicated in the usual manner.

Preparation of Antigen by Methods Other than the Routine Procedure

We have demonstrated the presence of complement-fixing antigen in extracts of infected brains of guinea pigs, prepared according to Howitt's (9) modifications of the technique of Craigie and Tulloch (25), which consists of the extraction of dried brain with ethyl-ether, resuspension of the non-extractable residue in physiological saline solution followed by repeated freezing and thawing in a salt-ice mixture, and removal of coarse particles by centrifugation. At times, however, this procedure did not yield detectable antigen and on no occasion was the serologically active substance present in large amounts.

It seemed likely that in the usual method of preparation of splenic extracts a considerable amount of antigen was lost in sedimented tissue which was discarded after the original horizontal centrifugation. Consequently, horizontal sediments were resuspended in the original volume of fresh serum-saline solution, frozen in a mixture of salt and ice, and subsequently thawed. Alternate freezing and thawing were repeated 5 or 6 times, after which the suspensions were carried through the regular procedure, *i.e.*, horizontal and ultracentrifugation, and Seitz filtration. On two occasions, extracts prepared in such a manner had a titer approximately equivalent to that of corresponding extracts prepared according to routine. However, on one occasion, only a comparatively small amount of antigen was obtained from the reclaimed sediment.

It became of interest to determine the largest amount of complement-fixing antigen which could be extracted from infected spleens and to compare it with the amount present in the regular preparations.

Accordingly, 10 infected spleens were divided longitudinally; one set of halves was ground and suspended in a volume of 30 cc. of diluent and prepared according to routine. The titer of the complement-fixing antigen in this extract was 1:16. The 10 remaining halves of spleens were ground in a mortar, without abrasive, suspended in sufficient saline solution to enable the material to be transferred to a tube, and desiccated *in vacuo* from the frozen state. The dried material, 0.2 gm., was resuspended in 20 cc. of saline solution and repeatedly frozen and thawed. After horizontal centrifugation, the supernatant suspension had a complement-fixing titer of 1:32. Thus, on the basis of dry weight, each gram of spleen yielded 12,000 fixing doses of antigen when treated according to the routine method, while 16,000 fixing doses were extracted by the modified procedure.

It is apparent from the above experiment that extracts of infected spleens, prepared according to the method we have usually employed, contained only a part of the complement-fixing antigen which could be obtained by another method.

DISCUSSION

The present observations indicate that a specific soluble substance separable from the virus of lymphocytic choriomeningitis plays the chief rôle in the complement-fixation reaction which occurs when extracts of infected tissue are mixed with immune sera. The slight fixation displayed by washed virus may be due to adsorbed soluble antigen. In certain virus diseases it has been suggested that the soluble antigen derives from the host instead of the infectious agent (26); the most suggestive example is the soluble substance found in yellow fever (2). On the other hand, the available evidence (27) regarding the soluble antigens of vaccinia indicates that they are intimately associated with the virus and are not derived from the host. At present it is not possible to give an opinion concerning the origin of the complement-fixing substance of lymphocytic choriomeningitis. However, it may be pointed out that, as in vaccinia, the antigen of lymphocytic choriomeningitis parallels in amount the infectious agent and is present irrespective of the species of animal infected, *i.e.*, guinea pig, mouse, or monkey.

Observations on the characteristics of the soluble antigen of this disease will be published later. Solutions of the antigen remain active for long periods of time when stored at ice box temperature, and they do not become anticomplementary. This stability facilitates the use of the soluble antigen in a diagnostic test which we have employed with serum from human beings suffering from lymphocytic choriomeningitis (10). Our results with patients' sera are in agreement with those of Lépine, Mollaret, and Sautter (24) who used for their antigen suspensions of consolidated lung of guinea pigs which undoubtedly contained both specific soluble substance and virus.

SUMMARY AND CONCLUSIONS

The virus of lymphocytic choriomeningitis can be sedimented in the ultracentrifuge and washed repeatedly; the virus retains its activity provided that a small amount of normal serum is present in the diluent.

A soluble substance capable of fixing complement in the presence of immune serum can be separated from the virus. Washed virus fixes complement poorly.

The serologically specific soluble antigen is widely distributed in tissues of infected guinea pigs, mice, and monkeys.

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DIURNAL VARIATIONS OF HEMOGLOBIN IN THE BLOOD OF NORMAL MEN

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The occurrence of diurnal variations of hemoglobin in the blood of normal people has been reported by a number of observers. Dreyer, Bazett, and Pierce (1) and Rabinowitch (2) have made determinations of the hemoglobin content of the blood of normal adults during the day and find a possible variation of 20 to 30 per cent of the average content. Short (3) finds a possible variation of 17 per cent as a result of his experiments. The methods employed by these investigators have not been so precise as those now available. The colorimetric methods used by Dreyer and his coworkers and by Short do not allow for the effect of variations in substances other than hemoglobin, such as lipids, which could affect the light absorption of the samples. Rabinowitch used the oxygen capacity method in his experiments, but employed an earlier and considerably less exact technique; also the inhalation of small amounts of CO by smokers may cause an appreciable alteration of the apparent hemoglobin content. His maximum variation during the day was more than double that observed by us.

Methods

The subjects in these experiments were healthy men from 20 to 30 years of age. They comprised laboratory technicians and medical students who were performing ordinary routine work. Blood was obtained by venipuncture from the arm veins at 2 to 3 hour intervals throughout the day. Precautions were taken to avoid stasis. The hemoglobin content was estimated from the CO-combining capacity according to the method of Van

Slyke and Hiller (4). In this technique the blood sample mixed with water is saturated with CO in the 50 cc. chamber of the Van Slyke manometric apparatus. Subsequently the excess CO and the O₂ and N₂ extracted by the shaking are expelled from the chamber, and the HbCO is determined by measurement of the CO set free by acid ferricyanide solution.

We used samples of 1 cc. of blood, and measured the pressure of the CO gas at 1 cc. volume. (A Van Slyke-Neill chamber calibrated at 1.0 cc., in addition to the usual 0.5 and 2.0 cc. points, was used.) These conditions gave a desirable combination of accuracy with economy of blood. The mean difference between duplicate analyses was 0.06 volume per cent of CO capacity.

The CO capacity method has over the O₂ capacity two advantages with respect to immunity from error.

1. The correction for physically dissolved O₂ is replaced by a smaller and practically constant correction for physically dissolved CO. According to the solubility determinations of Sengdroy, Dillon, and Van Slyke (5) normal blood saturated with air at 20° under 760 mm. of atmospheric pressure dissolves 0.70 per cent of O₂, and in blood of abnormal cell content the dissolved O₂ varies with the cell content. In comparison the correction for CO dissolved in the blood plus 2.5 volumes of water is only about 0.33 volume per cent of the blood in the technique of Van Slyke and Hiller, and is relatively independent of the cell content.

2. If the blood contains any preformed CO, as from tobacco smoke or the exhaust of automobiles, the O₂ capacity values are lowered, while the CO capacity is unaffected. The reason for this is that the CO is so strongly bound to the hemoglobin that agitation with air for the periods ordinarily used fails to replace the HbCO entirely with HbO₂. The O₂ subsequently determined in such a case therefore does not indicate all the Hb present. That the amount of CO in the blood of modern man can suffice to lower significantly the O₂ capacity values is indicated by the data on p. 112 of Enghoff's (6) monograph. He found O₂ capacities significantly lower than CO capacities in the bloods of seventeen out of 168 subjects; in these seventeen subjects the CO capacity exceeded the O₂ capacity by from 0.4 to 1.7 volume per cent.

TABLE I
*Hemoglobin Concentrations of Normal Young Men at Different Hours
of the Day*

Subject	Cc.CO capacity for 100 cc. blood								Range of day above and below median	Range of day in per cent of median
	9 a.m.	11 a.m.	2 p.m.	5 p.m.	8 p.m.	11 p.m.	Average for subject	Median for day		
W. D.	20.63	20.99	20.88	20.79	19.68	19.19	20.39	20.09	±0.90	±4.5
G. M.	20.03	19.00	19.26	19.24	18.93	18.46	19.16	19.25	±0.79	±4.1
F. W.	20.32	20.02	19.92	19.61	18.95	19.52	19.73	19.62	±0.70	±3.6
J. K.	18.84	19.97	18.82	19.27	18.12	18.45	18.92	19.05	±0.92	±4.8
F. R.	19.62	19.73	20.13	20.19	19.30	20.75	19.97	20.03	±0.72	±3.6
H. G. T.*	17.21	16.67	17.16	17.01	17.08	16.70	16.99*	16.94*	±0.27	±1.6
F. B.	19.88	19.45	19.90	18.89	19.21	18.86	19.38	19.90	±0.55	±2.8
J. F.	20.77	20.88	20.47	21.37	20.36	20.31	20.70	20.84	±0.53	±2.5
J. M.	20.56	20.72	20.59	19.92	19.86		20.33	20.29	±0.43	±2.1
J. B.	21.13	20.54	21.13	20.07	21.02	20.19	20.68	21.13	±0.53	±2.5
A. C.	21.63	20.33	19.36	19.52	19.59	19.31	19.95	20.50	±1.14	±5.4
J. S.	20.16	20.35	20.04	19.53	19.02	19.22	19.72	19.69	±0.67	±3.4
F. P.	19.38	18.93	18.83	19.14	18.87	18.61	18.96	19.00	±0.39	±2.0
R. S.	18.59		17.77	17.03	17.63	17.46		17.81	±0.78	±4.4
	18.65	18.23	17.93	18.26	18.63	18.24	18.02	18.29	±0.36	±2.0
D. K.	20.57	20.04	20.03	20.30	20.34	20.62		20.33	±0.30	±1.5
	21.30	21.06	20.55	20.39	20.31	20.04	20.48	20.67	±0.63	±3.1
E. J.	20.67		19.61	19.67	19.43	19.69		20.05	±0.62	±3.1
	20.62	20.07	19.38	19.33	19.25	18.99	19.70	19.81	±0.82	±4.1
R. E.	20.24	21.33	20.86	21.06	21.70	21.17		20.97	±0.73	±3.5
	21.48	21.92	21.31	21.41	21.20	20.70	21.20	21.31	±0.61	±2.9
L. A.	20.78	21.35	19.95	20.81	20.15	19.62		20.49	±0.87	±4.2
	19.87	20.00	19.63	19.52	19.34	19.01	20.02	19.51	±0.50	±2.6
Maximum.....							21.20	21.31	±1.14	±5.4
Minimum							18.02	17.81	±0.27	±1.6
Mean.....							19.86	19.94	±0.64	±3.2
Standard deviation $\sqrt{\Sigma d^2/n}$							±0.74	±0.92	±0.21	±1.0

* The exceptionally low data for H. G. T. are excluded in calculating means, minima, and standard deviations for "average for subject" and "median for day."

Results

The results from eighteen subjects are shown in Table I. In thirteen of the subjects the observations covered 1 day each; in

five subjects observations were made on 2 separate days about a week apart. Fig. 1 illustrates the changes observed in the five subjects who provided two curves each. From Fig. 1 there seems to be a tendency for the blood of each subject to follow a daily course typical of the individual.

The greatest range between the highest and lowest values for

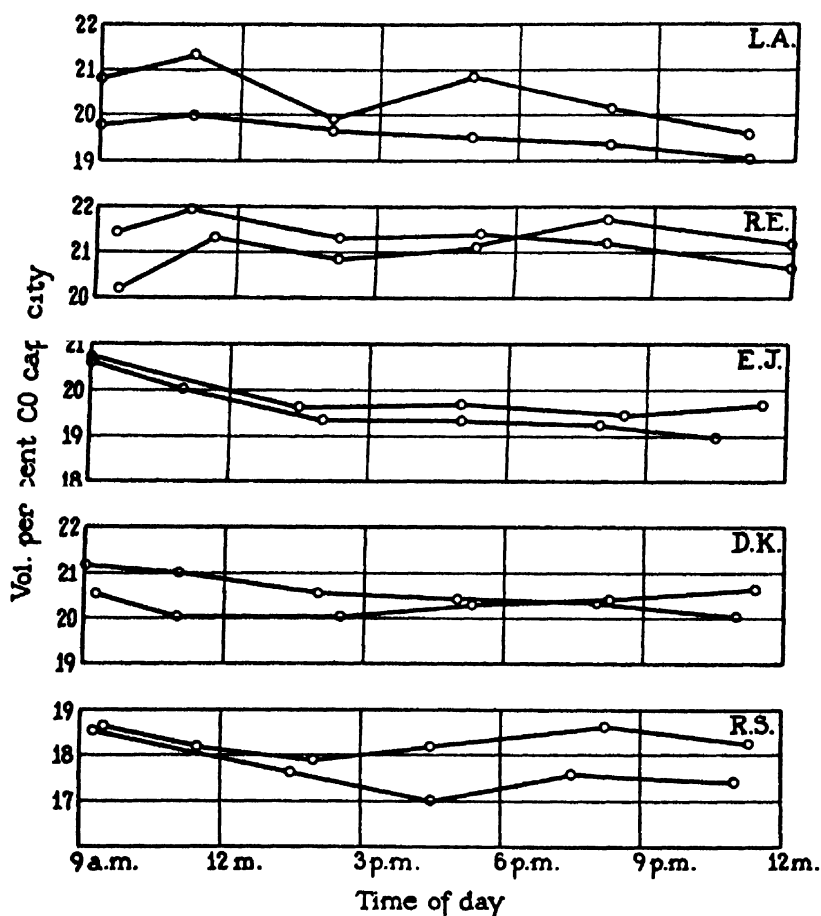


FIG. 1. Diurnal hemoglobin variations observed on 2 different days, about 1 week apart, in five young men.

1 day was 2.3 volumes per cent of CO capacity, or 11 per cent of the mean value. The average range was 1.3 volumes per cent of CO capacity, or 6.4 per cent of the mean.

These ranges of variation are narrower than those reported with earlier, and probably less exact, methods for hemoglobin measurement.

The direction of change in hemoglobin concentration during the day is not predictable from our data. There seems to be a tendency for the concentration to decrease during the waking hours: in 20 of the 23 days observed the hemoglobin at 11 in the evening was less than at 9 in the morning. The variation in behavior is such, however, that on a given day one could not predict even the direction of the change. Thus subject R. E. showed, from the earliest to the latest blood sample on 1 day, a decrease from 21.48 to 20.70, and on another day an increase from 20.24 to 21.17 volumes per cent.

The mean hemoglobin content of our eighteen subjects is represented by 19.9 volumes per cent of CO capacity. It appears that most of our subjects were in the lower half of the range, $20.7 \pm$ about 2.0, for normal men, which was estimated from data available in 1930 by Peters and Van Slyke (7), and has been confirmed recently by Enghoff's (6) precise study, with the CO capacity method applied to 95 men between 18 and 59 years of age.

SUMMARY

In eighteen young men hemoglobin determinations by Van Slyke and Hiller's manometric CO capacity method were performed at intervals of 2 to 3 hours from 9 a.m. to 11 p.m. on 1 or more days. The accuracy is indicated by a mean difference between duplicate analyses of 0.06 volume per cent of CO capacity.

The range between highest and lowest values in an individual through a day averaged 1.3 volumes per cent; the greatest range seen in any of the 23 observed days was 2.3 volumes per cent, equivalent to 11 per cent of the mean hemoglobin content for the day. Usually the hemoglobin content was lower in the evening than in the morning; but a subject showing this change on one day reversed it on another.

The much greater ranges of diurnal hemoglobin variation reported in the past literature appear attributable to methods of analysis which provided a significant part of the variations.

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OSMOTIC PRESSURE STUDY OF PROTEIN FRACTIONS IN NORMAL AND IN NEPHROTIC SUBJECTS

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This paper presents studies of protein osmotic pressures aimed to yield evidence on two questions, namely: (1) Whether albumin and globulin in the serum of patients with nephrosis are identical with albumin and globulin in normal serum, and (2) whether the albumin and globulin in the urine of such patients are identical with the corresponding proteins in the serum, either of normal subjects, or of the patients themselves.

1. Differences between Serum Proteins of Normal Subjects and Serum Proteins of Patients with Nephrosis

Earlier studies, reviewed by Goettsch and Reeves (6), and Alving and Mirsky (4), failed to reveal essential differences, either chemical or physicochemical, between normal serum proteins and the proteins in Bright's disease.

More recently, however, evidences of differences have been obtained. Working with the ultracentrifuge, McFarlane (11) has observed an abnormal sedimentation rate with the serum and urine from several cases of proteinuria, including nephritis, which suggested the presence of abnormally polydisperse albumin. As concerns nephrosis in particular, Tuchman and Sobotka (15) have found that the serum albumin contains more tyrosine, the globulin less, than in normal cases; Alving and Mirsky (4) have presented evidence of the existence of an abnormal albumin fraction with a low cystine content; Goettsch and Reeves (6) have observed immunological differences consisting in the fact that nephrotic albumin and globulin fail to precipitate completely with antisera developed against normal albumin and globulin.

Methods

Conditions for Constancy of Protein Osmotic Pressure Measurements.

—In protein solutions the specific protein osmotic pressure (pressure per unit weight of protein) is constant only at high dilution (1,3). At such dilution the law of van't Hoff relating osmotic pressure to molecular concentration appears to be valid for proteins, since the molecular weights calculated from the pressures agree with those by other physicochemical methods (13). At concentrations over 10 or 20 gm. per liter the specific osmotic pressure of the plasma proteins increases with increasing concentration. The cause of this phenomenon is not entirely certain. Adair and Robinson (3) conclude that Donnan's law does not explain it. The question has been reviewed recently (13). The essential fact is that to yield exact results pressure measurements must be made on solutions dilute enough to avoid the deviation from van't Hoff law.

If serum is diluted enough to make the proteins conform to van't Hoff's law, and if the albumin and globulin fractions have each the same mean molecular size in the serum as in preparations of these proteins separated by salting out methods, the osmotic pressure of the serum should accord with the equation:

$$P = 25 \left(\frac{C_a}{W_a} + \frac{C_g}{W_g} \right) \quad (1)$$

P is the osmotic pressure of the diluted serum in centimeters of water; 25 is the osmotic pressure of a millimolar solution of a non-electrolyte in water at 20°; C_a and C_g represent the concentrations, in mg. per liter, of albumin and globulin, respectively, and W_a and W_g the molecular weights calculated from the specific pressures of separated albumin and globulin. Adair and Robinson (3) have shown the basic validity of this equation by finding good agreement between the osmotic pressures of unfractionated serum at infinite dilution and those calculated from the partial pressures of albumin and globulin, using the molecular weights which they had found with preparations of separated albumin and globulin.

Pressure Measurements.—An apparatus has been recently described (5) which makes the accurate determination of low osmotic pressures a rapid and easy procedure. The amount of protein necessary for

one determination is only 0.2 cc. and its concentration need not be higher than 0.2 per cent. At such low concentrations, the van't Hoff law can be applied without corrections; therefore the results have been expressed, for simplicity's sake, directly as molecular weights.

The procedure previously indicated has been closely followed (5). Except in Table I, nearly all determinations were carried out in duplicate, sometimes triplicate or quadruplicate. There were practically no erratic results. Duplicate determinations usually checked within less than 5 per cent, the mean deviation for all the determinations being less than 2 per cent. The molecular weights given in the tables were calculated with the help of Table I in the preceding publication (5). The lower the concentration, the more closely (excluding experimental errors) should the figures given represent the true molecular weights. Actually, in the range of small concentrations used here, the differences are usually unappreciable.

Preparation of Material.—For the preparation of dilute serum samples (Table II), the blood was simply allowed to clot at room temperature, the cells were discarded after centrifugation, and serum and diluting fluid were mixed in the proportions indicated in Table II. Albumin and globulin concentrations were determined by Howe's precipitation and Van Slyke's manometric micro Kjeldahl method (12).

For the preparation of the protein fractions, it was felt that the simplest procedure would be the best, since it was the least likely to interfere with the state of aggregation of the proteins. In this instance there seemed to be no point in subjecting the material to such drastic treatments as have been applied by some investigators (17), especially since even the most elementary procedure of precipitation has been shown by the ultracentrifuge to cause definite irreversible changes (10), and since our aim for the moment was to find whether there were differences between normal and pathological sera, rather than to isolate more or less artificial products with apparently constant properties.

The procedure, which was entirely conducted at room temperature, was generally as follows:

The blood was allowed to clot and the cells discarded after centrifugation. To about 5 cc. of serum was added an equal volume of saturated ammonium sulfate

solution, and the precipitate formed was filtered off after a few hours. The globulin precipitate was washed several times on the filter with half saturated ammonium sulfate, then scraped from it and dissolved in a little water. Since with nephrotic globulins the solution remained as opaque as milk, it was then in some cases shaken twice with an equal volume of ether, which was syphoned off after centrifugation. This procedure left a practically clear solution, which was transferred into a cellophane bag for dialysis. The albumin was precipitated from the filtrate with an excess of solid ammonium sulfate, filtered off, and then transferred. An alternative procedure, which was successful with nephrotic serum only, was to centrifuge the precipitated albumin. It would then collect rapidly at the top of the tube like a thick yellow paste, and could be scooped up with a spatula, the remaining fluid being water-clear. Apparently the high lipid content of the albumin fraction in nephrosis is responsible for this behavior, since in normal cases centrifugation at usual speed is quite ineffective.

In some cases the procedure was slightly varied. In one case, the serum albumin was caused to crystallize at room temperature by adding $M/1$ acetic acid to the filtrate from globulin. The crystals were kept in the ice box over 2 months in the mixture recommended by Adair and Robinson (2) before they were dialyzed. In another case, normal globulin scraped from the filter was dissolved in 15 cc. of $0.15\ M$ NaCl, then reprecipitated at half saturation before dialysis. Still in another, normal plasma instead of serum was used for a globulin determination. Details of technique are briefly indicated in the tables.

Dialysis was performed with small sections of cellophane tubing clamped flat against a piece of hard rubber plate and rocked in a trough for a few hours; the outer fluid, $0.15\ M$ NaCl, was often renewed and the gradual decrease in it of ammonia concentration could be easily traced with Nessler's reagent. For the preservation of globulin solutions, a higher concentration was found preferable, therefore concentrated NaCl was added after dialysis so as to make the final salt concentration about 0.9 molar.

It should be clearly understood that the terms albumin and globulin used here mean nothing more than two rather easily separable fractions, and that no claim is made as to their individuality or homogeneity. By definition, the two fractions obtained in this way from normal serum represent what is usually understood as albumin and globulin, the characters of both of which are now, from the physico-chemical standpoint, fairly well established; but in connection with nephrotic serum these two words are used here only in their restricted original sense, namely, to designate, respectively, that part of serum (or urine) protein which precipitates at complete saturation, and that part which precipitates at half saturation, with ammonium sulfate.

When normal serum is used, the separation of the two fractions in this way is quite sharp, the filtration can be carried out immediately, and the filtrate containing the albumin fraction remains clear indefinitely. The procedure was found to be less satisfactory when dealing with nephrotic serum, though filtration could be performed more rapidly than with Howe's method (9); the filtrate obtained was usually quite transparent after an hour or two, but would not remain so more than 12 or 24 hours.

The protein solutions obtained after dialysis sometimes contained a very slight precipitate which could be filtered off easily; in case of normal albumin and urine proteins, the filtrates were water-clear; with pathological sera and normal globulin, a slight milkiness usually persisted, even after treatment with ether. Treatment with ether did not appreciably affect the osmotic pressures measured.

The nitrogen content was determined by Van Slyke's gasometric Kjeldahl method (12). For the protein:nitrogen ratio, the factors 6.41 for albumin and 6.61 for globulin, obtained by Adair and Robinson (3) for horse serum, were provisionally used.

The nephrotic subjects from whom the serum was obtained were as follows:

G. B., female, 24 years, typical nephrotic syndrome of 1 year's duration. About 35 liters of edema in November, reduced to 15 liters 2 months later. Proteinuria: 30 gm. per day. Subnormal urea clearance.

S. G., male, 9 years, typical nephrotic syndrome of 1 year's duration. Considerable ascites and edema. Proteinuria: 6 gm. per day. Normal urea clearance.

P. F., male, 3 years, and W. H., female, 33 years; both cases of nephrotic syndrome with low urea clearances.

Results of Serum Protein Studies

Table I gives the molecular weights calculated from the osmotic pressures of normal human albumin and globulin. For albumin, the figure of 72,000 may probably be taken as a reasonable average, and it appears that the mode of precipitation had no effect on the results. For globulin, the figures given for Jan. 11 should probably be chosen as the most reliable; each one of them is the average of four determinations (in each case two osmometers were used and the determinations repeated on the same sample of serum). The most trustworthy osmotic pressure measurements obtained from

animal serum yield molecular weights of about 72,000 for albumin and 170,000 or 175,000 for globulin (13); it appears that the values

TABLE I

*Molecular Weights of Normal Human Albumin and Globulin
Calculated from Observed Colloidal Osmotic Pressures**

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin

Subject	Albumin		
	Concentration	Molecular weight	Remarks
	<i>per cent</i>		
H. F. Oct. 1	0.342	71,600	After one crystallization
J. B. Nov. 28	0.215	71,800	Direct dialysis of albumin filtrate after globulin precipitation
	0.430	72,000	
	0.645	69,600	
	0.320	71,400	Albumin precipitated from filtrate before dialysis
	0.374	71,600	
	Globulin		
Dec. 31	2.08	162,000	One precipitation. Shaken with ether
	1.04	164,000	
	0.502	157,000	
Jan. 11	1.52	165,000	Two precipitations. Not shaken with ether
	0.760	163,000	
Dec. 9	0.615	183,000	From oxalated plasma

* Calculated by the formula

$$\text{Mol. wt.} = \frac{p_s}{p} \times c \times 10^6$$

p_s = pressure of a 0.1 mM solution at the T° of the experiment.

p = pressure of the unknown protein solution.

c = concentration of the protein solution in grams per cent.

(Cf. preceding publication (5) Tables I and II.)

for man are not appreciably different. Ether extraction of lipids had no effect on the results. In one case plasma instead of serum

was used, and the mean molecular weight found for the globulins was significantly higher. If one assumes that fibrinogen represents one-tenth of the total globulins, a rough calculation indicates that its molecular weight would have to be about twice that of the other globulins to cause the observed difference between plasma and serum; the point is left open for further investigation.

Table II gives the osmotic pressures developed by diluted serum both in normal and in nephrotic cases, and the theoretical pressures calculated with the help of equation 1 by taking 28.8 cm. of toluene as the pressure of a millimolar solution at 21°, assuming all the sera investigated to be a mixture of albumin with a molecular weight of 72,000 and of globulin with a molecular weight of 164,000, and taking for the concentrations the values indicated in the second column. Various diluting fluids were used, and the uniformity of the results shows that neither the salt content nor the pH of the fluid had any significant effect on the pressures developed. It appears that, whereas in the normal cases observed osmotic pressures agreed closely with those calculated from equation 1 with the molecular weights found for normal proteins, such agreement was not found for the nephrotic sera. The mean deviation of +4 per cent found with normal serum may be easily accounted for by the possibility that the albumin concentration is actually a little greater than the values obtained by Howe's method (9). The mean deviation of -35 per cent in the nephrotic cases indicates the presence of proteins with abnormally high molecular weights.

To ascertain whether the nephrotic proteins were in fact of abnormally high molecular weight, the osmotic pressures of albumin and globulin isolated from nephrotic sera were measured. The results, expressed as mean molecular weights, are given in Table III. The weights found for albumin are about 50 per cent higher, for globulin 100 per cent higher or more, than in normal cases. Above 2 per cent concentrations, the deviation from the van't Hoff law already becomes apparent, the molecular weights obtained being appreciably smaller than in more diluted solutions.

If we take the case of G. B., Jan. 10, (Table II) and substitute in equation 1 the values obtained on that day for albumin and globulin concentrations and those of Dec. 31 for the molecular weights (Table

TABLE II
Osmotic Pressure of Highly Diluted Human Serum
Temperature = 21°C.

Subject	Original concentration	Diluting fluid	Dilution	Pressure observed mm. toluene	Pressure calculated mm. toluene	Deviation		
	<i>per cent</i>					<i>per cent</i>		
<i>Normal</i> J. B. Nov. 12	A = 5.31 G = 2.19	0.15 M NaCl	1:11	24.2	22.8	+6		
			1:22	12.3	11.4	+8		
			1:22	12.0	11.4	+5		
		M/15 Sørensen's phosphate; pH = 7.65*	1:11	23.0	22.8	+1		
			1:11	24.3	22.8	+7		
		M/15 Sørensen's phosphate; pH = 5.88*	1:11	23.5	22.8	+3		
			1:11	23.5	22.8	+3		
		0.1 M Na acetate 0.1 M acetic acid; pH = 4.64*	1:16	15.8	15.7	+1		
			1:16	16.0	15.7	+2		
		Nov. 23	A = 5.13 G = 2.77	0.12 N NaCl 0.03 N NaHCO ₃ + CO ₂ †	1:11	24.3	23.1	+5
1:22	11.7				11.5	+2		
<i>Nephrotic</i> G. B. Nov. 16	A = 1.37 G = 1.89	0.15 M NaCl	1:11	5.2	8.0	-35		
			1:11	5.1	8.0	-36		
		M/15 Sørensen's phosphate; pH = 7.7‡	1:11	5.3	8.0	-34		
			1:11	5.3	8.0	-34		
		0.1 M Na acetate 0.1 M acetic acid	1:11	4.9	8.0	-39		
			1:11	5.1	8.0	-36		
		Nov. 22	A = 1.61 G = 2.09	0.12 N NaCl 0.03 N NaHCO ₃ + CO ₂ †	1:11	5.3	9.2	-42
					1:4	17.2	25.2	-32
		Jan. 10	A = 1.57 G = 2.15	0.15 M NaCl	1:4	16.5	25.2	-35
					1:8	8.5	12.6	-33
1:8	8.4				12.6	-33		
1:8	8.2				12.6	-35		
S. G. Nov. 30	A = 0.82 G = 2.98	0.15 M NaCl	2:7	15.0	24.4	-39		
			2:7	15.5	24.4	-36		

* Determined with the glass electrode after dilution.

† This mixture was made by bubbling expiratory air through the solution.

‡ Calculated value before dilution.

III), the theoretical pressure for a serum diluted four times would be (taking 28.8 cm. of toluene as the pressure of a mM solution at 21°)

$$\left(\frac{15,700}{102,000} + \frac{21,500}{240,000} \right) \times 28.8 \times 1/4 = 17.6 \text{ mm. of toluene,}$$

instead of the 16.5 mm. observed. For a dilution of $\frac{1}{8}$ the calculated values would be 8.8 mm. instead of the 8.2 to 8.5 observed.

TABLE III

*Molecular Weights of Serum Albumin and Globulin in Nephrotic Subjects
Calculated from Colloidal Osmotic Pressures*

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin.

Subject	Albumin		Globulin	
	Concentration	Molecular weight	Concentration	Molecular weight
	<i>per cent</i>		<i>per cent</i>	
G. B. Nov. 27	0.166	105,000		
	0.196	106,000		
Dec. 31	0.442	104,000	2.13	217,000
	0.628*	104,000	1.07	240,000
	0.314*	99,000		
S. G. Nov. 29	0.196	122,000		
Dec. 16			1.34	298,000
Dec. 31			2.17	314,000
			1.09	353,000
			0.504	346,000

* In these two samples the lipids were extracted with ether after dialysis, after which the sample was redialyzed for 2 hours.

More accurate calculations are probably not warranted, since the proteins were precipitated with ammonium sulfate for the molecular weight determinations, and with sodium sulfate for the determinations of concentration. The two salts have been found to precipitate approximately the same amounts of globulin in normal serum, but whether the same holds for nephrotic serum has not been investigated.

2. Nature of the Proteins in the Urine of Nephrotic Patients

The earlier literature about proteinuria has been reviewed by Hiller *et al.* (8), who have found that in nephrosis the albumin:globulin ratio is usually above 10, whereas it is usually lower in glomerulonephritis. It has been thus far generally assumed that the albumin and globulin of urine in Bright's disease were identical with the albumin and globulin of normal serum. McFarlane (11), however,

TABLE IV

*Molecular Weights of Urine Albumin and Globulin in Nephrotic Subjects
Calculated from Colloidal Osmotic Pressures*

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin

Subject	Albumin		Globulin	
	Concentration	Molecular weight	Concentration	Molecular weight
	<i>per cent</i>		<i>per cent</i>	
S. G. Dec. 1	0.560 0.187	61,700 62,000		
G. B. Dec. 5	0.439	61,600	1.24 0.620	114,000 120,000
Jan. 23			0.712	107,000
P. F. Dec. 5	0.555	66,700		
W. H. Dec. 12	0.403	57,200	1.13	120,000

has observed that the urine albumin of patients with Bright's disease is less homogeneous in the ultracentrifuge than is normal albumin.

Methods

For the preparation of albumin and globulin fractions urine was treated like serum, except that treatment with ether was omitted. The albumin-containing filtrate, after globulin precipitation, was always perfectly clear, as that of normal serum. Osmotic pressures were measured as indicated above.

Results with Urine Proteins

Table IV gives the results. The molecular weights calculated from the osmotic pressures of the urine albumin and globulin are definitely below the weights obtained for the albumin and globulin of normal serum; the difference is more marked for globulin than for albumin.

DISCUSSION

It goes without saying that the molecular weights given for albumin and globulin fractions in the tables should be assumed to indicate only the average sizes of the molecules in each fraction. The globulin fraction, from its salting-out curves, electrophoretic behavior (14) etc., is in general believed to include more than one distinct protein, and the albumin fraction of nephrotic serum appears also to be heterogeneous. Of the fractions here studied, the albumin of normal serum is the only one that, from its physicochemical behavior and its crystallizability, may perhaps be homogeneous. We have not been able to crystallize the albumin of nephrotic patients, either from serum or from urine.

McFarlane (11) has studied the sedimentation rates in the ultracentrifuge of the serum and urine proteins of five cases of proteinuria. One of these was apparently a case of nephrosis, another a case of nephritis. In all cases the serum showed the presence of polydisperse albumin. Our conclusions are therefore in accord with his.

The data of Table II indicate that the pressure of highly diluted serum can be expressed as the sum of the partial pressures of albumin and globulin, if correct figures for the specific pressure, or molecular weight, of each fraction are used in the calculation. This appears to be true not only for normal serum, but also for nephrotic serum, in which the molecular weights of both albumin and globulin, as separated with ammonium sulfate, were found to be from 50 to 100 per cent higher than in normal serum.

Since we are still in ignorance as to the laws which govern deviation from the van't Hoff law, it is probably not warranted to compare the results presented here with those obtained from osmotic pressure measurements on higher concentrations, and without attempt to estimate specific pressures at infinite dilution. This statement applies to the formulas, calculated by Govaerts and his followers (7, 16, 18),

relating the osmotic pressures of undiluted serum to its albumin and globulin concentration, and also to Widdowson's results (17).

The results of Table IV show that the urine proteins of the nephrotic patients differ from serum proteins of the same patients even more markedly than from the serum proteins of normal subjects. Roughly, the mean molecular weight of urine albumin is one-half that of serum albumin, that of globulin one-third the molecular weight of serum globulin, from the same patient.

These results support the idea that in proteinuria the kidney is more permeable to the proteins of smaller molecular size. It has long been known that albumin passes more abundantly than globulin into the urine (8). Our results indicate that, from the heterogeneous albumin fraction of nephrotic serum, the subfractions of lower molecular size pass more abundantly into the urine; and similarly for the subfractions of the globulins.

SUMMARY

In serum of patients with nephrosis both albumin and globulin showed by osmotic pressure nearly double the molecular weights of normal albumin and globulin.

In the urines of such patients, on the other hand, both proteins showed molecular weights lower even than in normal serum.

The colloidal osmotic pressures were measured by the author's method at such dilutions that the van't Hoff law relating pressures to molecular concentrations could be directly applied. For the albumin and globulin of normal serum the molecular weights found were 72,000 and 164,000 respectively, in agreement with the weights obtained by other methods.

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EFFECT OF PARTIAL CLAMPING OF AORTA IN DOGS UPON DIASTOLIC PRESSURE IN CAROTID AND FEMORAL ARTERIES

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It is well known that in the human congenital anomaly, coarctation of the aorta, both *systolic* and *diastolic* levels of pressure are frequently elevated in the arteries proximal to the constriction.^{1, 2} It has recently been suggested that interference with the blood supply to the kidneys is the mechanism underlying the development of the arterial hypertension found in this condition.

The evidence that this is so is that arterial pressure rises in the upper half of the body only when the clamp upon the aorta is placed above the site of origin of the renal arteries. Rise in mean pressure in the carotid artery was studied by direct measurements in dogs³ and increase in pressure in rats^{4, 5} was inferred from the weights of the hearts at death being distinctly greater when the aortas were clamped above the origin of the renal arteries than when they were clamped below or not clamped at all.

If the mechanism of the development of hypertension occasioned by clamping the aorta resembles that which follows clamping the renal arteries there should occur an increase in diastolic arterial pressure throughout the body in parts distal, as well as proximal to, the constriction of the aorta. So far, diastolic pressure has not been measured in experimental work nor were pressures in the hind extremities measured. Yet in human cases of coarctation of the aorta collected by King² the data suggest and, more recently direct measure-

¹ Lewis, T., *Heart*, 1933, 16, 205.

² King, J. T., *Ann. Int. Med.*, 1937, 10, 1802.

³ Goldblatt, H., and Kahn, J. R., *Proc. Cent. Soc. Clin. Res., J. Am. Med. Assn.*, 1938, 110, 686.

⁴ Rytand, D. A., *Proc. Soc. Exp. Biol. and Med.*, 1936, 38, 10.

⁵ Rytand, D. A., *J. Clin. Inv.*, 1938, 17, 391.

ment of pressure in the femoral artery in a case⁶ shows, that elevation of diastolic pressure may be present in the lower extremities.

In the present study observations were made of the changes in diastolic pressure in the legs of three dogs following partial clamping of the aorta above the origin of the renal arteries. The experiments were carried out as follows: Van Leersum loops⁷ were made of one carotid artery. Pressures were then recorded in both the carotid

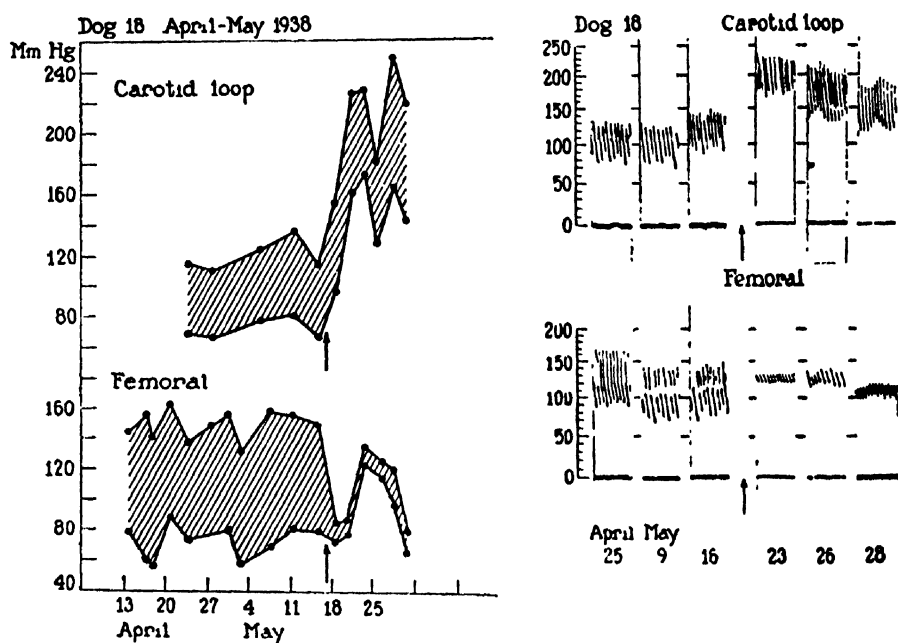


FIG. 1

Chart of carotid and femoral arterial pressures from dog No. 18 read from records obtained by use of Hamilton's intra-arterial manometer. At the right (Fig. 1a) are reproduced a few of the original records. The black arrows indicate application of the aortic clamp on July 7.

and femoral arteries by means of Hamilton's intraarterial manometer⁸ about 4 times a week. The frequency and sensitivity of the manometer were, of course, such that both systolic and diastolic levels of pressure were accurately recorded. Relatively constant levels be-

⁶ Steele, J. M., and Cohn, A. E., *J. Clin. Inv.*, 1938, **17**, 514.

⁷ Van Leersum, E. C., *Arch. ges. Physiol.*, 1911, **142**, 377.

⁸ Hamilton, W. F., Woodbury, R. A., and Harper, H. T., *Am. J. Physiol.*, 1934, **107**, 427.

came established in 3 or 4 weeks. The animals were then anesthetized with pentobarbital and an adjustable metal clamp was placed upon the aorta above the renal arteries, but below the coeliac axis and adrenal arteries. Some interference with the adrenal blood supply occurred, in all probability, in most of the animals but it was obviously not sufficient to interfere perceptibly with the function of the glands. The aorta was gradually compressed by means of the clamp while pressure in the femoral artery was continuously recorded

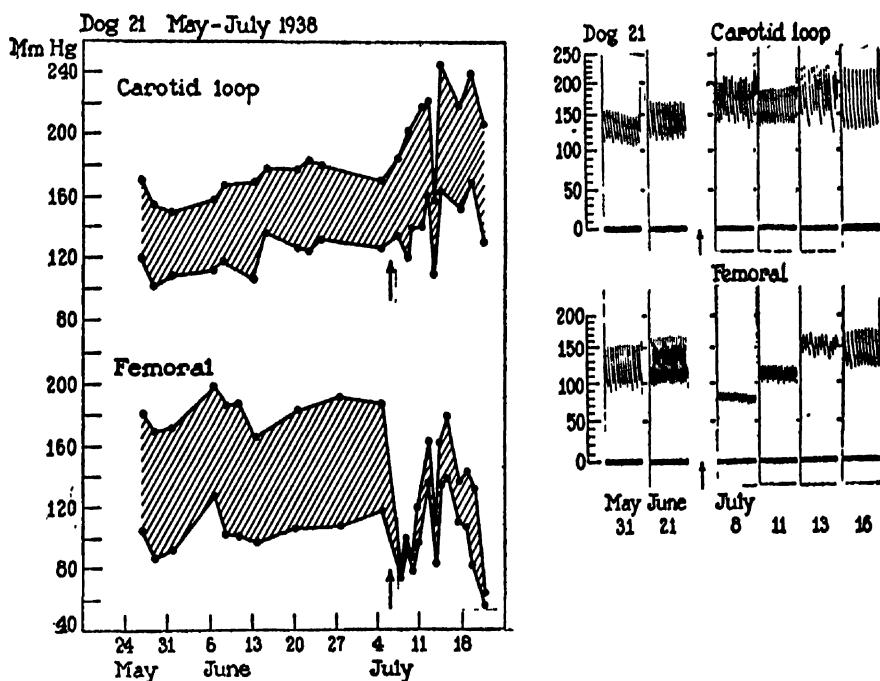


FIG. 2

Same as Fig. 1, for dog No. 21. The clamp was applied on May 17.

until pulsation had almost disappeared. The clamp was then fixed in this position. At this point little fall in diastolic level occurred, but if an attempt was made to obliterate the pulse altogether, the diastolic pressures fell abruptly. After securing the clamp the wound was closed. Records of pressure were obtained after operation almost daily for a period of about 2 weeks.

Within 24 hours both systolic and diastolic pressures in the carotid artery rose (Figs. 1 and 2). During the next 72 hours both levels of pressure in the femoral artery began also to rise from the very

low postoperative levels occasioned by constriction of the aorta, and the pulse pressure, though still small, increased. Within 5 days the *diastolic* pressure in the lower extremities rose to a level plainly higher than the preoperative one and remained elevated for about a week, or until slow hemorrhage into neighboring tissues took place through erosion of the aorta by the clamp.

The diastolic level in the hind legs does not, of course, always rise. Whether it does seems to depend upon the degree of occlusion of the aorta. If the aorta is completely occluded, and if sufficient collateral circulation fails to develop, as occurred in one instance, the pressure in the hind legs remains low though both pressures rise in the carotid artery; but if it is insufficiently clamped neither the pressures in the legs nor those in the carotid artery rise. The marked reduction in flow to the lower extremities seemed in the instance of complete occlusion responsible for the failure of the pressure to rise; the hind limbs were cold and failure of nutrition began before the animal was killed.

From these experiments the conclusion can be drawn that clamping the aorta in dogs above the orifices of the arteries to the kidneys may be followed by diastolic hypertension in the hind legs as well as in the neck. Increase in peripheral resistance is widespread. Elevation of pressure does not depend therefore upon mechanical factors alone as in acute experiments when the aorta is occluded (Barcroft⁹). Nor can the hypertension in coarctation of the aorta be explained by local mechanical factors such as the increase in resistance offered by the narrowed aorta and the collateral paths around it.¹⁰ It depends upon a reaction of the whole peripheral arteriolar system. Hypertension, the result of clamping the aorta, is in this respect similar to that following constriction of the renal vessels. In both cases the deciding factor is interference with the dynamics of the renal circulation.

One further remark seems pertinent. The observation of similar consequences to the arterial pressures of constriction of the aorta in man (coarctation of the aorta) and in dogs (clamping of the aorta) warrants the inference that interference with the hemodynamics of

⁹ Barcroft, H., *J. Physiol.*, 1931, 71, 281.

¹⁰ Blumgart, H. L., Lawrence, J. S., and Ernstene, A. C., *Arch. Int. Med.*, 1931, 47, 806.

the renal blood supply in man may lead to arterial hypertension as Goldblatt has demonstrated that it does in dogs and monkeys.

Summary. Clamping the aorta above the orifices of the renal artery in dogs is followed by elevation of the diastolic level of arterial pressure in the hind legs as well as in the carotid arteries. Constriction of the peripheral arterioles is, therefore, a general phenomenon, just as when it follows partial clamping of the renal arteries. The hypertension which develops in coarctation of the aorta in man is on this evidence analogous to that which accompanies constriction of the renal arteries. The evidence suggests strongly that interference with the hemodynamics of the renal circulation leads to hypertension in man as well as in animals.

METABOLISM OF LEUCOCYTES IN RINGER-PHOSPHATE AND IN SERUM

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Ponder and MacLeod^{1, 2} described the oxidative rate of leucocytes obtained from peritoneal exudates in rabbits, but no studies of glycolysis were made, and Ringer-phosphate solution was the only medium employed. In this communication are presented the results of studies of aerobic and anaerobic glycolysis of exudate leucocytes, and a simple method by which their respiration in serum may be measured is described.

Bakker³ stated that exudate leucocytes respired at a low rate ($QO_2 = 0.4$) but the rate of aerobic glycolysis was high ($Q_G^{O_2} = 6$). Fleischmann and Kubowitz⁴ reported rates 10 times as great ($QO_2 = 4$) for the respiration of similar cells, and figures for aerobic and anaerobic glycolysis of 11 and 21 respectively. Both authors used Ringer-phosphate solution as a medium. Fujita⁵ using rat blood leucocytes suspended in citrated rat plasma reported a high respiratory rate ($QO_2 = 9$) and a low rate of aerobic glycolysis ($Q_G^{O_2} = 2$).

The experiments of Bakker and of Fleischmann and Kubowitz have been repeated and the results are here reported. The leucocytes of peritoneal exudates induced in the rabbit were suspended in Ringer-phosphate solution. The technic for obtaining the cells has been described.^{1, 2} The cell suspension used contained about 70,000 cells per mm³, and 2 cc of the suspension were sufficient to allow good measurements of the metabolic rate over a period of 3 hours.

¹ Ponder, E., and MacLeod, J., *J. Gen. Physiol.*, 1936-37, **20**, 267.

² Ponder, E., and MacLeod, J., *Am. J. Physiol.*, 1938, **123**, 420.

³ Bakker, G., *Klin. Woch.*, 1927, **6**, 252.

⁴ Fleischmann, W., and Kubowitz, F., *Biochem. Z.*, 1927, **181**, 395.

⁵ Fujita, A., *Klin. Woch.*, 1928, **7**, 897.

Respiration was measured in Ringer-phosphate at a pH of 7.3, in an atmosphere of pure O_2 . For aerobic glycolysis, the cells were suspended in Ringer-glucose-bicarbonate, and the measurements made after equilibration with 95% O_2 and 5% CO_2 . For anaerobic glycolysis the cells were suspended in the same medium equilibrated with 95% N_2 and 5% CO_2 . The results express the average metabolic activity during the first hour in 10 experiments.

QO_2	$Q_G^{O_2}$	$Q_G^{N_2}$
4.6	17	25

These figures are slightly higher than those of Fleischmann and Kubowitz, but are in the same range. We cannot confirm the low rate of respiration reported by Bakker. The cause of the abnormally high rate of aerobic glycolysis observed is obscure. It is possible that the effectiveness of respiration in preventing aerobic glycolysis has been damaged in these cells, either by the methods used in obtaining them, or by the use of Ringer-phosphate solution as the suspension medium.

The effect of serum on the respiration of exudate leucocytes has been investigated. Normal serum cannot be used in the presence of KOH because of the marked pH changes due to the loss of CO_2 . It is possible to modify rabbit serum so that the bicarbonate content is reduced and the resulting pH change in the presence of KOH is relatively small. The method of preparation is as follows: 15 cc of serum are titrated with N/10 HCl until the pH is about 6.4. In the case of normal rabbit serum which has been exposed to the air for some time from 1.5 to 2 cc of N/10 HCl are required. The acidified serum is then thoroughly evacuated until the pH reaches 7.3. Measured quantities are then transferred to the Warburg vessel, the cells added, and respiration measured in the conventional manner in an atmosphere of O_2 , KOH being used to absorb CO_2 . In this way, special apparatus is not necessary.*

* Dr. C. O. Warren, Jr., has applied the neutralized serum technic to the respiration of bone marrow and compared the results obtained with those of experiments done in untreated serum in the Dixon-Keilin manometer; he found no appreciable difference in the QO_2 values in both systems though both showed a marked increase (50%) over that of Ringer-phosphate (private communication).

In 12 experiments the respiration of exudate leucocytes has been measured in neutralized serum. In all the respiration was greater than in Ringer-phosphate. This increase varied from 35 to 70% with a mean of 50%. The mean QO_2 in neutralized serum is 7 as compared to a QO_2 of 4.6 in Ringer-phosphate. Furthermore, maximum respiratory activity is maintained longer in serum than in Ringer-phosphate. The serum alone consumes sufficient O_2 to account for not more than 15% of the increase.

In several control experiments the change in pH of the neutralized serum in the manometer in the presence of KOH for a period of time equivalent to the usual duration of an experiment has been measured. The changes were from 0.2–0.3 toward the alkaline side (7.3–7.6). In the case of exudate leucocytes where aerobic glycolysis is of considerable magnitude this change of pH is offset by acid production. The pH of the cell-serum system after 3 hours in the manometer in presence of KOH falls from 7.3 to approximately 6.9.

Summary. The rates of respiration and aerobic glycolysis of exudate leucocytes reported by Fleischmann and Kubowitz have been confirmed. A method of measuring the respiration of leucocytes in serum is described. The respiratory rate of leucocytes in serum is greater than the rate in Ringer-phosphate solution.†

† We are greatly indebted to Dr. E. Shorr of the New York Hospital for much valuable advice concerning the use of serum.

STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

II. PATHOGENESIS OF THE DISEASE IN THE GUINEA PIG

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PLATE 40

(Received for publication, January 5, 1939)

In the first paper of this series (1), the histopathology of equine encephalomyelitis in the guinea pig was described and discussed in detail. The virus injected peripherally readily invades the blood stream and then infects the central nervous system. The mode of entrance of the virus into the nervous system from an injection site at the periphery is not clear.

Hurst (2), studying the guinea pig, believed that the local peripheral nerves are not involved in the pathogenesis. He suggested that virus is deposited from the blood on the olfactory mucosa, whence it travels to the subdural space by means of the perineural lymphatics. The recent work of Yoffey and Drinker (3) makes such an explanation improbable. Hurst also suggested the passage of virus from the blood across the endothelium of the cerebral blood vessels (in his phrase, a "growth through" the hematoencephalic barrier).

Sabin and Olitsky (4) believed that, in contrast to the behavior in mice, the virus in the guinea pig passes directly across the vascular endothelium, since in this animal the lesions bear a definite relation to blood vessels. With the Western strain of the virus, Larsell, Haring, and Meyer (5) similarly suggested a direct passage across the blood vessels, also on the basis of the perivascular nature of the lesions. The validity of this evidence will be treated in the discussion.

Because of the lack of unequivocal data, the problem was reinvestigated. Topographical analysis of early cases seemed the most promising method. This method depends on the assumption that the earliest localization of the virus will produce the first lesions. Although, as is well known, virus may be present without causing tissue damage, an area with a demonstrable typical lesion presumably has harbored the virus for a longer period than any area without lesions.

By charting all the lesions in a brain the earliest localizations can thus be determined.

Method

Since topographical analysis would be valueless when the encephalitis is fully developed, it was essential to secure very early cases for study. To ensure the presence of lesions before the brains were serially sectioned, a method of vital staining was used, modified from that of McClellan and Goodpasture (6). The following procedure was used in guinea pigs. 1 to 2 hours before the animal is to be sacrificed, 2 to 3 cc. of 2 per cent trypan blue are injected intravenously. It is usually desirable to administer an additional 1 cc. subcutaneously the day previously. The intravenous injection usually prostrates the animal which, after 1 to 2 hours, is killed with chloroform. The nervous system is then perfused with formalin through the aorta under physiological pressure. When the brain is removed, lesions are clearly outlined in blue against the colorless background. The entire success of the method depends on securing a good perfusion. Otherwise even the normal portions of the brain may be colored, due to dye contained within the blood vessels.

With this method, animals were killed at various intervals after inoculation. The nerve tissue was imbedded in paraffin and sectioned serially at 15 microns. Every 15th or 20th section was mounted and stained.

Two strains of virus were used. One was isolated in 1937, and the 2nd, 3rd, and 4th subinoculations in guinea pigs were employed. The 2nd was isolated in 1938, and the 1st guinea pig subinoculation was used. The results of the two strains were indistinguishable.

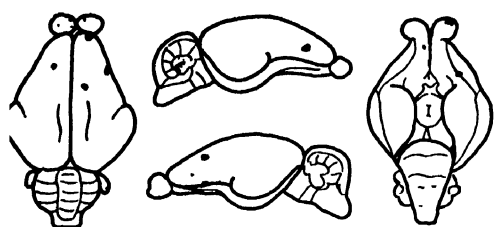
OBSERVATIONS

Observations on 9 selected animals, none of which showed any clinical signs of disease at the time of sacrifice, are presented in detail. Even a cursory survey of the material shows that, with a constant mode of inoculation, the lesions are never the same in any two cases. In the accompanying figures are charted the lesions which appear on the surface. All those which are hidden from view in the interior of the brain are described in the accompanying text. Summaries are presented in Table I and should be consulted in conjunction with the charts. For a detailed description of the anatomical structures mentioned below, reference may be made to standard works on the nervous system of rodents (7).

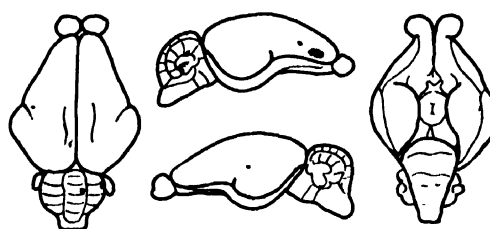
No. 1. Inoculation into both hind pads. Sacrificed after 58 hours. Every 15th section mounted and stained.—Both olfactory bulbs contain a few small discrete

TABLE I

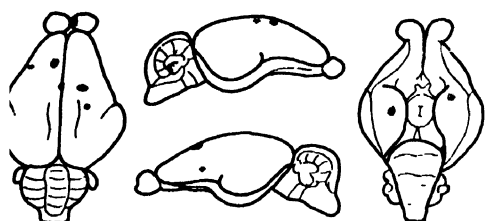
Guinea pig No.	Route of inoculation	Killed (time after inoculation)	Summary of lesions
		hrs.	
1	Hind pads	58	Small discrete lesions scattered over the frontal part of the brain, involving the olfactory and neocortical portions about equally. Similar lesions present in the cerebellum. Subcortical centers intact
2	" "	57 $\frac{3}{4}$	Scattered lesions present in neocortex and cerebellar cortex, as well as in a few unrelated subcortical centers
3	" "	58 $\frac{3}{4}$	Scattered lesions involving portions of the olfactory brain and unrelated areas in the neocortex. Subcortical lesions situated only in the intermediate auditory centers; lowest, primary auditory nuclei normal
4	" "	62 $\frac{1}{4}$	Cerebral lesions restricted to extensive involvement of the olfactory regions, without affecting the hippocampus Isolated lesions in the cerebellar cortex
5	" "	58 $\frac{1}{2}$	Inflammatory involvement primarily of the lower and higher visual centers of both sides, more severe on the left. Most of the thalamus, including the non-visual portions, affected, as well as certain auditory centers. Parts of the olfactory pathways, as well as the basal ganglia, also injured
6	Intranasal	67	Widespread lesions in lower olfactory connections; but circumscribed, unrelated lesions also present in neocortical areas, the caudate nucleus, the cerebellum, and the mid-brain
7	Intraocular (right)	66	Involvement of right anterior olfactory regions, and of scattered neocortical areas. Thalamus and subcortical centers intact
8	" "	58	Involvement of entire optic pathway of both sides, from the chiasm to the cortex, the left side more than the right. Lesions extend to contiguous areas which are functionally independent. Isolated lesions present in the left claustrum and pons, regions independent of each other and of the affected portions elsewhere. Certain intermediate acoustic centers affected
9	" "	58 $\frac{1}{2}$	A few scattered lesions in the intermediate olfactory centers and in the neocortex. Olfactory bulbs normal. Lower visual centers corresponding to the inoculated eye show very early involvement, but of much less intensity than the cortex



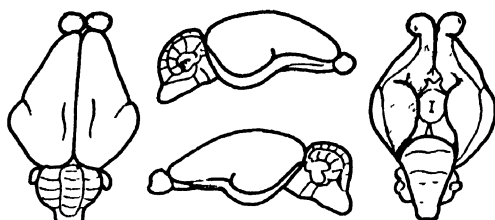
TEXT-FIG. 1. Guinea pig 1.



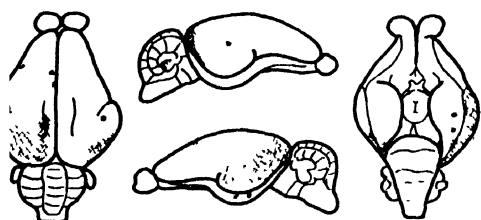
TEXT-FIG. 2. Guinea pig 2.



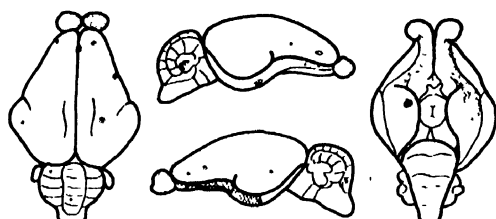
TEXT-FIG. 3. Guinea pig 3.



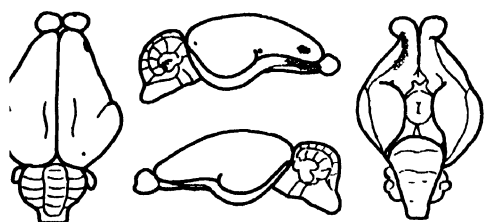
TEXT-FIG. 4. Guinea pig 4.



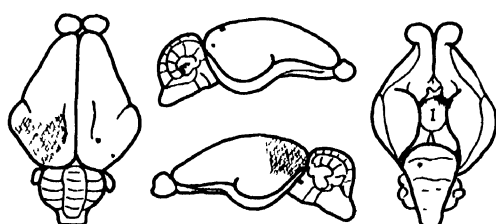
TEXT-FIG. 5. Guinea pig 5.



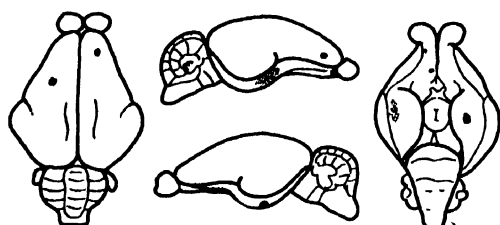
TEXT-FIG. 6. Guinea pig 6.



TEXT-FIG. 7. Guinea pig 7.



TEXT-FIG. 8. Guinea pig 8.



TEXT-FIG. 9. Guinea pig 9.

lesions. In the remainder of the olfactory brain there are only two other lesions, one in the anterior olfactory nucleus on the right, the other astride the rhinal fissure on the left, involving the neo- and olfactory cortex about equally on either side of the fissure. There are other lesions in the neocortex, as indicated in the chart, affecting the orbital, precentral, postcentral, and insular areas. There are no concealed lesions; the hippocampus, basal ganglia, thalamus, midbrain, pons, and medulla are entirely normal, as is the spinal cord. In the cerebellum there are a few sharp foci, two of which, in the flocculi, show exquisite bilateral symmetry.

No. 2. Inoculation into both hind pads. Sacrificed after 57½ hours. Every 15th section mounted and stained.—No part of the olfactory system is involved. In the neocortex lesions are present in the right postcentral area, involving the insular cortex to some extent, and the right parietal region. There is a small focus of low intensity in the left temporal cortex. Two isolated lesions are present in the cerebellar cortex. Several lesions are present in lower centers, not indicated in the chart. There is bilateral ependymitis with, in one area, a collection of polymorphonuclear leucocytes impinging on the ventricle. The left caudate nucleus also contains a small but intense focus of inflammation. The right medial geniculate body and the right inferior colliculus contain early lesions, but the acoustic tubercles and intermediate centers are entirely normal. No other lesions are present in any portion of the brain or cord. In the meninges are two small areas, one in the left temporal cortex and one at the base at the level of the posterior thalamus, where there is some mononuclear infiltration, slight in both degree and extent. These meningeal areas are not related to regions of parenchymatous inflammation.

No. 3. Inoculation into both hind pads. Animals sacrificed 58½ hours later. Every 15th section mounted and stained.—In the entire olfactory system there are only three lesions, one in the medial portion of the right bulb and one in the entorhinal region of either side. The intermediate centers are intact, as is the hippocampus. Most of the lesions are situated in the neocortex where the left precentral and parietal, and the limbic or infraradiar areas are involved. On the right the lesions affect the parietal and the most anterior portion of the striate areas. Hidden from view are early lesions in the right inferior colliculus and right medial geniculate body. No other portion of the thalamus shows any injury. The lateral geniculate, sending fibers to the striate cortex, and the anterior nuclei, which send afferents to the limbic area, are intact. The medullary acoustic centers, along with the entire medulla, cerebellum, and cord, are normal.

No. 4. Inoculation into both hind pads. Sacrificed after 62½ hours. Every 20th section mounted and stained.—The olfactory bulbs of both sides contain numerous minute foci of polymorphonuclear leucocytes. There is in addition much more extravasation of blood than is usually seen. The olfactory centers at the base of the brain are affected in very widespread fashion. On the right practically the entire extent is involved by innumerable small foci, sometimes discrete, sometimes confluent, of varying degrees of intensity. On the left the process is similar but essentially limited to the anterior pyriform cortex, sparing the tuberculum

olfactorium. The process extends inward to a slight extent, to include the amygdala and the nucleus accumbens. Although the presubiculum is damaged, the involvement does not include the hippocampus. For the most part the inflammatory changes are sharply limited dorsally by the rhinal fissure, but in a few areas there is a very slight extension into the adjacent neocortex. The only other sign of encephalitis in the entire brain is the presence of three isolated discrete foci of inflammation in the cerebellar cortex. Thalamus, midbrain, pons, and medulla are intact.

No. 5. Inoculation into both hind pads. Sacrificed after 58½ hours. Every 20th section mounted and stained.—The olfactory bulbs are intact. There is a minute lesion in the left olfactory crus, in the dorsal part of the left anterior olfactory nucleus adjacent to the orbital cortex. There are also two discrete foci of destruction in the left entorhinal cortex. Apart from a single isolated focus in the left precentral area, the other neocortical lesions are restricted to the striate, temporal, and occipital areas. On the right there is a single circumscribed lesion in the visual area, and a more diffuse region of injury more posteriorly, which overflows into the inferior temporal cortex and the subiculum. On the left, however, the damage is quite extensive, involving the greater part of the visual and posterior temporal cortex, and also overflowing into the subiculum. It will be noticed that the chiasm is intact, but the optic tracts exhibit small discrete lesions, more on the left.

Hidden from view there is rather widespread damage to the telencephalon and diencephalon. Thus, the left caudate nucleus contains a small focal collection of polymorphonuclear leucocytes. The left putamen and globus pallidus and the amygdala more posteriorly also contain early lesions. In the thalamus there are inflammatory changes involving the anterior group, the lateral, and the ventral nuclei, on the left. The medial as well as the lateral geniculate bodies of both sides are severely injured, as well as the pretectal regions and the posterior nuclei. There is a much milder involvement of the medial nuclei of the thalamus, the subthalamus, and portions of the hypothalamus. The changes in the superior colliculi are well marked; those in the inferior colliculi are very mild, with the right somewhat greater than the left.

The white matter of the hemispheres, including the thalamic peduncles, shows very numerous glial nodules, and some cuffing of the blood vessels. The cerebellum, pons, and medulla, as well as the hippocampus, are normal. There are no changes in the medullary acoustic centers. The spinal cord is normal.

No. 6. Intranasal instillation, both nostrils. Sacrificed at 67 hours. Every 20th section mounted and stained.—The maximum involvement affects the basal olfactory centers. The bulbs are the seat of numerous small lesions, which, on the left, continue backward in a rather diffuse fashion, including the anterior olfactory nucleus and pyriform cortex. The tuberculum olfactorium is bilaterally spared. The lesions diminish in intensity posteriorly and disappear in the position marked. On the left the process is much less severe, only the anterior pyriform cortex being affected. More posteriorly is an isolated lesion as marked. The

hippocampus is entirely spared. In the neocortex there are numerous discrete circumscribed lesions situated in diverse architectonic areas—precentral, postcentral, parietal, insular, temporal, and striate. The cerebellar cortex also displays two isolated lesions.

Hidden from view there is a mild degree of ependymitis of the left lateral ventricle. The left caudate nucleus contains a small inflammatory focus. The right lateral geniculate body, which sends fibers to the striate cortex, is intact; but the right superior colliculus, which merely receives from the cortex without sending, shows very early changes. Both inferior colliculi show extensive but very early involvement, the right somewhat more than the left. But the medial geniculate bodies, as well as all the medullary acoustic centers, are completely normal.

No. 7. Intraocular inoculation into the right eye. Sacrificed after 66 hours. Every 20th section mounted and stained.—The right olfactory bulb shows a few small scattered lesions. On the same side the anterior olfactory nucleus and the anterior pyriform lobe show well marked inflammatory changes. In the neopallium there are three discrete lesions, one in the right postcentral cortex, and one in the right temporal cortex. There is in addition a small focus of neuronal degeneration in the right occipital cortex. The thalamus and other subcortical centers are entirely normal.

No. 8. Intraocular inoculation into the right eye. Animal sacrificed 58 hours later. Every 20th section mounted and stained.—The olfactory bulbs and entire anterior portion of the brain, including neocortex, olfactory cortex, and anterior basal ganglia, are entirely free of lesions. The first lesions are seen in the optic chiasm and optic tracts, where the left is more severely affected than the right, although both contain glial and leucocytic foci. The hypothalamus and olfactory regions adjacent to the ascending optic tract on the left show well marked inflammatory reaction. The surface lesions are restricted to the striate and occipital cortex on the left, where the involvement is widespread. On the right, there are only two discrete focal lesions in the striate cortex. In addition there are seen lesions in the left midbrain (nucleus of the lateral lemniscus) and in the right pons, both reaching the surface of the brain. Hidden from view are lesions in both lateral geniculate bodies and both superior colliculi, the left being more severely damaged than the right. Both medial geniculates are involved, but the inferior colliculi are intact. The damage to the midbrain and posterior thalamus is not restricted to the nuclei mentioned, but tends to extend medially in a somewhat diffuse fashion. There is an isolated circumscribed lesion in the left claustrum at the level of the infundibulum. The medulla, cerebellum, and cord are entirely normal. The medullary acoustic centers show no change.

No. 9. Intraocular inoculation into the right eye. Sacrificed after 58½ hours. Every 20th section mounted and stained.—The olfactory bulbs are free of lesions although there are discrete scattered lesions more posteriorly in the rhinencephalon, namely, small early foci in the right anterior pyriform cortex and the right tuberculum olfactorium. More posteriorly there is a well circumscribed

focus in the left entorhinal cortex and a more diffuse area of involvement in the comparable region on the right side. In the neocortex there are lesions in the right precentral and insular areas, and also in the right postcentral and parietal areas. In the subcortical centers, hidden from view, the left lateral geniculate and the left anterior colliculus show extremely early but very definite lesions. The remainder of the thalamus is entirely intact, as is the hippocampus, as well as all other portions of the brain.

General Features

It is of interest to observe whether virus lesions are isolated or whether they tend to involve systems. Of the 9 cases presented, in 4 there is definite system involvement, although of different sense modalities: the olfactory pathway in Nos. 4 and 6, and the visual pathway in Nos. 5 and 8. It is noteworthy that different routes of inoculation were utilized. In line with this, it is seen that the same route of inoculation can, in different instances, produce totally different distribution of lesions (as No. 8 contrasted with No. 9, both with intraocular injections; or, with pad inoculation, No. 4 contrasted with No. 2). On the other hand, as already mentioned, different modes of inoculation can produce substantially the same result.

Apart from the occasional system involvement, there should be noted the frequency of discrete scattered lesions in the cortex, of the hemispheres, the cerebellum, and the olfactory brain. The virus appears to attack the brain not only with reference to nerve paths, but also independently of known system connections.

It is of further interest that although the cortex is the site of predilection, subcortical centers may also be affected at times. Thus, various regions in the basal ganglia, thalamus, hypothalamus, and midbrain show damage in one or more instances. It is important to realize that no antipathy exists between the subcortical regions and the virus.

Evidence for Nerve Transmission

The inference that the nerve pathway is implicated in pathogenesis depends for its force on two requirements. First, the number of lesions in the brain must be small. Where the entire nervous system is a mass of inflamed tissue, no differential significance can be attached to the involvement of any particular part. Second, the nerve centers

implicated must be connected by simple and direct nerve paths. A scheme whereby a circuitous nerve connection is imagined, involving several intermediate stations and tracts of doubtful existence, is ingenious but not convincing. The force of the "nerve path transmission," theory is proportional to the directness of the connecting pathway, and inversely proportional to the total number of lesions.

The best example of the importance of nerve connections in the spread of the virus is offered by No. 8. Here inoculation was into the eye, and the lesions involve, in a quite selective manner, the entire optic pathway up to the cortex. Other unrelated lesions will be discussed below. In No. 5, with virus injected into the pads, there is a strikingly similar distribution of lesions. In this latter case, the virus carried by the blood attacked some part of the optic pathway, and then spread out along the nerve paths of the visual system.

The utilization of existing nerve paths in the spread of virus within the brain is shown by the olfactory connections. Virus instilled into the nose (No. 6) affected the olfactory bulbs, the pyriform lobes, and entorhinal cortex, which are connected with the bulbs by the lateral olfactory tract. The other lesions will be discussed below. In No. 4, a pad inoculation, the bulbs and pyriform cortex are affected in a manner very similar to the previous case. Similarly in No. 7, the right olfactory bulb and the right anterior pyriform cortex both contain lesions. Here too the obvious link between the two involved regions is the nerve pathway.

These examples suffice to show that in certain instances, where there are relatively few lesions in the entire brain, anatomically related nerve centers may be involved in a quite selective and striking fashion. There are two possibilities: either pure coincidence has occurred, or the nerve connections have had some part in the spread of the virus. The latter choice alone seems plausible in the instances cited.

Evidence for Spread by Direct Contiguity

When two contiguous portions of the nervous system are involved, the relationship may be one of simultaneity and independence; or, as in the preceding section, a spread from one region to the other by means of nerve connections; or, third, direct extension from one area

to the other, as by passive transport of virus particles in the interstitial fluid.

It is very difficult to rule out possible nerve spread, for the short nerve connections of different regions of the brain are incredibly numerous. But in No. 3 a crucial example is presented, namely, the involvement of adjacent regions where nerve connections are known not to exist. The optic tract on the left is severely damaged, and the contiguous hypothalamus, medially, and amygdala, laterally, also show inflammatory changes. Neuronal relationship between the tract and these adjacent regions does not exist. The alternative of spread by direct extension appears overwhelmingly probable.

Many other probable examples exist in the foregoing protocols, but this mode of spread once established does not need elaboration.

Evidence for Blood Stream Transmission

The virus, once it has attained the central nervous system, may at times spread along the nerve processes or axones, that is, by nerve transmission; or it may spread by direct extension. But how does the virus reach the central nervous system following a subcutaneous inoculation?

In the present study the evidence for passage directly from the blood stream into the brain is the converse of that used to demonstrate nerve transmission. The latter evidence depends for its force on the involvement of successive nerve levels connected by known anatomical pathways. Conversely, the isolated involvement of a given center, all of whose known nerve connections are normal, is presumptive evidence that the nerve pathway is not involved. Blood stream transmission is the only reasonable alternative.

It is readily seen that in the brains previously described the majority of lesions occurred independently of nerve pathways. A few obvious examples will suffice. In Nos. 5 and 9, there are isolated lesions in various parts of the rhinencephalon, but the olfactory bulbs, where all incoming olfactory fibers end, are entirely normal. In Nos. 2 and 3, there are lesions in the acoustic centers of the midbrain and thalamus, yet the acoustic tubercles, where the peripheral nerves end, are normal. Brain 6 has a lesion in the striate (visual) area, but the corresponding lateral geniculate body is intact. Similarly

No. 3 has a well marked focus in the left limbic (infradiar) region, but the anterior nucleus of the thalamus, which is the afferent subcortical center, is unaffected. Brain 7 shows a massive focus of destruction in the postcentral (sensory) cortex, yet the entire thalamus is normal. Several brains show small foci in the cerebellar cortex, but the cerebellar connections are normal.

The frequency of discrete small lesions in the neocortex, together with the intact state of the subcortical centers, is readily apparent. The virus injected into the periphery appeared in the highest level of the nervous system without any evidence of having passed through the appropriate lower centers. To travel along nerve paths from the periphery to the cortex, certain primary and intermediate nuclei must be traversed. Severe lesions in the higher centers, with perfectly normal lower centers, would seem to exclude the utilization of nerve paths. The only reasonable alternative is that the virus attacked the cortex directly through the blood vessel wall.

The Rôle of the Spinal Fluid

Were the cerebrospinal fluid of significance in the spread of the virus through the nervous system, an entirely different histological picture would be called forth. It is known that this virus is capable of attacking the leptomeninges. Consequently a primary meningoencephalitis, with fairly uniform distribution of lesions at the margins of the spinal fluid pathway, would be expected. This has not been observed. Instead, sharply circumscribed focal lesions appear, not only on the surface of the brain, but also deep in the hemisphere and brain stem. There appears no way to harmonize these data with any possible rôle of the spinal fluid in the pathogenesis of this disease.

DISCUSSION

The importance of the blood in the pathogenesis of this disease is beyond question. The virus is known to circulate in high titer. We see further that after a constant mode of inoculation, as by pad injection, or intraocular inoculation, the distribution of lesions is inordinately varied. The blood stream is the only agent which could account for such vagaries.

This is of especial interest in relation to intraocular or intranasal

inoculation. Out of three cases of intraocular injection presented, in only one was there clearly an involvement of the optic system. Even though the virus may utilize nerve connections from the eye there is still an escape into the blood. Virus once in the blood acts essentially at random, regardless of the site of inoculation. In such instances as Nos. 6 and 8, the primary spread is related to the site of inoculation (nose and eye, respectively). But there are numerous totally unrelated lesions. These latter are attributable to secondary spread by way of the blood stream, and not by way of hypothetical nerve paths as yet undiscovered.

That the spread of viruses may be determined by existing nerve paths is an inference. This inference has been invoked by many authors in the study of different neurotropic viruses (8). In the present study of the guinea pig, the involvement of related nerve centers is sometimes extremely striking. For example, Nos. 4, 5, 6, and 8 show an involvement of successive neurones of a given system (olfactory or visual), with relatively little damage to the remainder of the brain. The probability is overwhelmingly high that this selective involvement was not merely due to chance: in some way the anatomical connections influenced the spread of the virus.

In certain other instances the probability of nerve spread is less strong—much less so. For example, in No. 3 there are a single lesion in the left olfactory bulb and single lesions in the posterior pyriform cortex, both right and left. There may be some causal connection between these three lesions; that is, the known nerve connections might be invoked as a connecting link. But there might, in this case, be a relationship merely of coincidence. If we compare the olfactory regions of Nos. 1 or 3 with those of Nos. 4 or 6, we see a marked difference. In the two latter instances, nerve spread seems definite; in the two former, it is problematic. Coincidence is equally or even more likely, especially in view of the spotty distribution of lesions elsewhere in the nervous system.

On the other hand, in this series the majority of lesions in the brain, especially in the neocortex, do not bear any relationship to each other according to known anatomical connections. In such instances a nerve spread of virus seems overwhelmingly improbable. The alternative of direct spread from the blood stream is the only satisfactory explanation.

There is only one criterion of nerve spread, namely, the topographical relationship of affected areas. Certain authors (4, 5) have held that the perivascular nature of some lesions is an indication that the virus passed through the blood vessels. In the present series of cases, where several hundred lesions were studied, this distinction is found not to obtain. No histological difference could be observed between regions where the virus was presumably blood borne, as compared with regions where nerve transmission presumably occurred. Only the anatomical relations of the affected regions, compared with each other and with the remainder of the nervous system, furnish a ground for distinction.

It is thus seen that the virus of equine encephalomyelitis is bipotential. It enters the brain from the blood stream through the blood vessel wall. Once in the nervous system, it may or may not spread along preexisting nerve paths. No explanation can at present be offered why in one instance it does and in another does not.

A question which must be kept open is that of possible deposition of virus from the blood on the olfactory mucosa, with subsequent spread along the olfactory connection (Hurst (2), Sabin and Olitsky (4)). The mere presence of lesions somewhere in the olfactory brain is obviously not sufficient to establish this theory. However, a marked involvement of the olfactory bulbs is entirely consistent with the hypothesis. In the present series of cases, No. 4 might be interpreted in this sense if one so desired. In the present state of our knowledge, this theory must be held under reserved judgment. There can be no doubt that in reference to certain neurotropic viruses the nose and the olfactory pathway stand in a different category from most other sense modalities and other parts of the body. The reason is as yet obscure, and further studies in anatomy as well as pathology are necessary.

The findings in the guinea pig may be compared with the results obtained by Sabin and Olitsky (4) in young mice, which alone were found susceptible to peripheral injection. According to these authors, the virus may occasionally invade the central nervous system along the local peripheral nerves. More often, they believe, virus transported by the blood invades by means of the olfactory or auditory pathways, or possibly along the seventh nerve. "No evidence was found of a direct passage of virus across the blood vessels of the brain" (9).

In the guinea pig, occasional animals show primary involvement of

the olfactory pathway, and there is no way to rule out a prior deposition of virus on olfactory mucosa. But in no other case was there evidence of spread *via* the peripheral nerves; that is, in no other case were the primary receptive nuclei involved. The cases of spread along the optic nerve are not exceptions, since the optic nerve is not a peripheral nerve at all. With the possible exception of the olfactory nerve, the virus seems to have attacked the central nervous system directly, without the intermediation of the peripheral nerves. This attack is considered to take place by passage across the blood vessels of the brain.

SUMMARY

After inoculation with equine encephalomyelitis virus by various routes, guinea pigs were sacrificed at early stages, before symptoms were apparent. The brains were studied histologically, with serial sections; all lesions were noted, and subjected to topographical analysis. Nine cases are presented in detail.

With any given mode of inoculation the distribution of lesions varied very widely from one instance to another. In some cases, affected regions bore a striking and definite anatomical relationship to each other. These distributions can be explained only by the assumption that the anatomical pathways played some rôle in the spread of the virus. In other instances lesions were present in areas, the anatomical connections of which were entirely normal. Attention is called to the frequency of lesions in the neocortex, with intact subcortical centers. Such distribution is held to render nerve spread extremely improbable. The only satisfactory explanation of such random distributions is by direct passage of virus from the blood stream into the brain tissue. There is no histological difference between lesions which result from blood spread and those resulting from nerve spread.

CONCLUSIONS

In the guinea pig, the virus of Eastern equine encephalomyelitis, injected peripherally, invades the blood stream and passes directly from the blood stream into the brain. This seems to be the principal, though not necessarily the exclusive, mode of pathogenesis. Once in

the nervous system the further spread of the virus may occasionally be determined by anatomical connections.

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EXPLANATION OF PLATE 40

FIGS. 1 and 2. Two typical lesions, from guinea pig 1. Thionin. Fig. 1, $\times 43$. Fig. 2, $\times 65$.

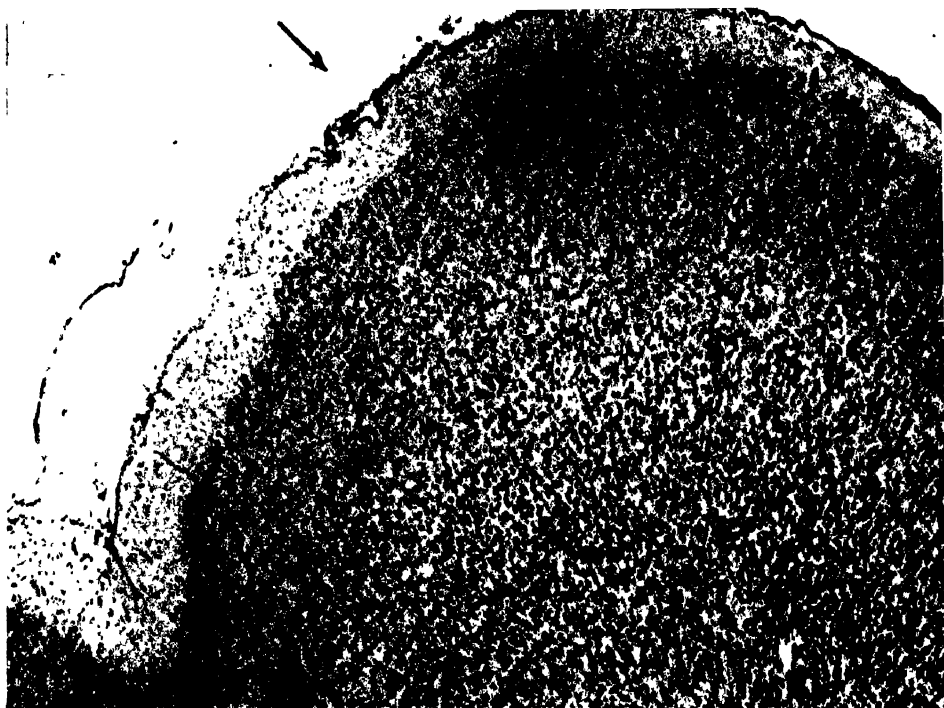


FIG. 1

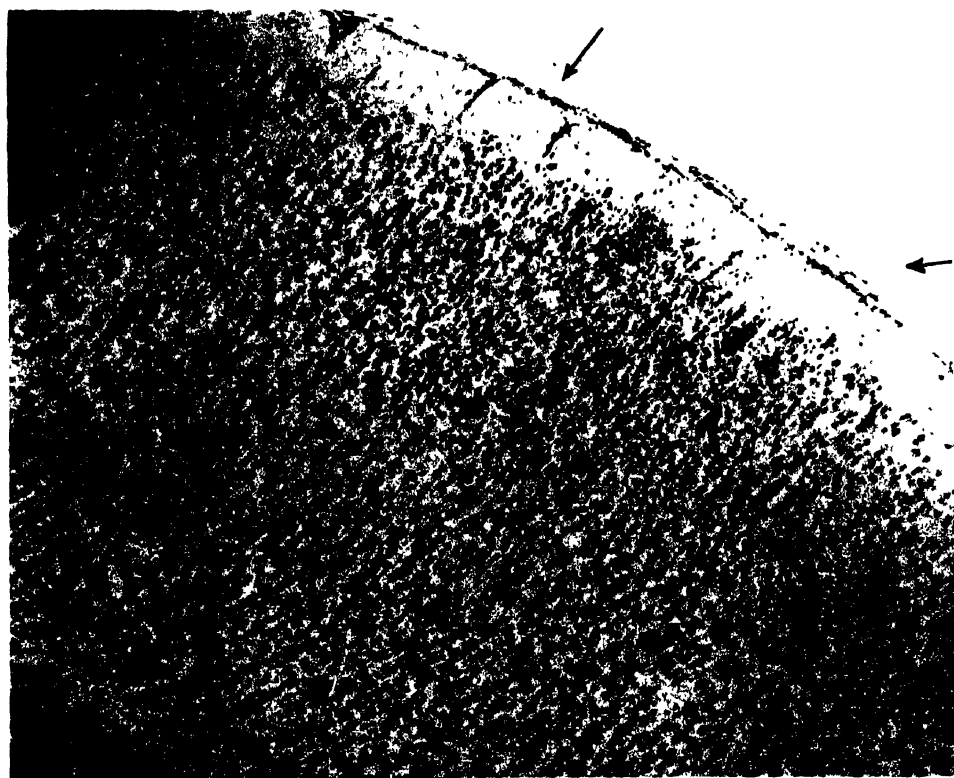


FIG. 2

Photographed by J. A. Carlile

STUDIES ON EASTERN EQUINE ENCEPHALOMYELITIS

III. INTRAOCULAR INFECTION WITH FIXED VIRUS IN THE GUINEA PIG

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PLATE 41

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In the preceding paper (1) it was shown that a recently isolated strain of equine encephalomyelitis virus, when injected into the eye, sometimes spread along the optic pathways. This type of spread was, however, inconstant. But with a fixed strain of virus (2, 3) an entirely constant behavior was observed which was capable of systematic study.

For the study of virus activity within the central nervous system, inoculation into the eyeball presents certain great advantages due to the anatomical peculiarities of this organ, which make it the most readily accessible portion of the central nervous system.

Anatomy.—In general, the peripheral and central nervous systems differ fundamentally in the type of accompanying stroma. Within the brain or spinal cord there are glial cells which form the supportive tissue, but no true connective tissue stroma. Small quantities of connective tissue accompany the blood vessels but are shut off from the true nerve parenchyma by the pia-glial membrane. On the other hand, in the cranial or spinal nerves, including the sensory ganglia, there is a rich stroma of fibroblasts and associated cells, collagen, and reticulin, with a true lymphatic system. The junction between the central and the peripheral portions of the craniospinal nerves has recently been well described by Tarlov (4). The importance of this stroma in relation to certain problems of the so called hemato-encephalic barrier has been emphasized in recent publications (5, 6).

The optic nerve is not a peripheral nerve at all, in the sense applied to the vagus, hypoglossal, or any of the spinal nerves, for example. Instead, the optic nerve is embryologically and anatomically a tract of the brain substance proper, having all the characteristics of the central nervous system.

It is well known, of course, that the optic pathway in the lower vertebrates is chiefly though not entirely crossed, the decussation taking place in the optic chiasm. The principal connections of the right eye, for example, are with the left lateral geniculate body, and pretectal nucleus of the thalamus, and the left superior colliculus. The converse obtains with the left eye. From the lateral geniculate body a projection tract goes to the striate cortex of the same side.

Method.—In the experiments herein reported, a 27 gauge needle was inserted from the inner canthus of the eye directly through the sclera into the posterior chamber of the eye. Passage through the anterior chamber was avoided. The injection mass (0.03 to 0.05 cc.) was placed in contact with the ganglion cells of the retina and the overlying fibers of the optic nerve.

The virus was thus placed in the vitreous humor, in contact with the cells and fibers of the central nervous system with essentially no trauma to the latter.

Strains of Virus.—For the most part a strain of Eastern equine encephalomyelitis virus that had been modified by serial intracerebral passage in pigeons was employed. This strain was developed by Traub and TenBroeck (2), and has been further studied in detail by Traub (3). In the present experiments the 112th to 117th pigeon passages were utilized. In addition certain comparative experiments were carried out with an unmodified strain of the Eastern virus, isolated from a horse in 1937, of which the 2nd to 5th intracerebral guinea pig passages were utilized. All titer figures refer to tenfold dilutions of a 10 per cent suspension of pigeon (or guinea pig) brain, which is designated as 10^0 dilution.

Sensitivity of the Intraocular Route

To determine the relative sensitivity of the intraocular as compared with the direct intracerebral route, comparative titrations were performed using the same amount of inoculum for each route. Both the fixed strain and the unmodified strain were used. The results of two such titrations are given in Table I. In some experiments fatalities have resulted from a 10^{-3} dilution of the fixed strain, and as high as 10^{-6} of the unmodified strain, injected intraocularly.

With the fixed strain there is a definite difference between the intraocular and the sub- or intracutaneous routes. Traub (3) and TenBroeck and Traub (2) have already found that guinea pigs are quite insusceptible to subcutaneous inoculation, and the present work fully confirms their results. With the dosage used (0.05 cc.) the undiluted virus suspension regularly failed to produce encephalitis following an intracutaneous injection, although the intracerebral titer was 10^{-6} or 10^{-7} (with the same dosage).

With the unmodified virus, on the other hand, repeated experi-

ments with the same quantity of inoculum did not reveal any significant difference between the intraocular and intracutaneous routes of administration.

With the fixed virus, it is clear that the susceptibility of the guinea pig to intraocular inoculation is midway between the intra- or subcutaneous and the intracerebral routes. This difference does not obtain with the unmodified virus.

TABLE I

Titration of Intraocular and Intracerebral Inoculations in Guinea Pigs

Dilution	Fixed strain (pigeon passage)		Unmodified strain	
	Intraocular inoculation	Intracerebral inoculation	Intraocular inoculation	Intracerebral inoculation
10 ⁻⁸	N.T.	N.T.	0, 0	0, 0
10 ⁻⁷	"	2, 0, 0	0, 0, 0	5, 9, 0
10 ⁻⁶	"	3, 0, 0	0, 0, 0	3, 4, 6
10 ⁻⁵	"	2, 3, 3	4, 6, 0	3, 3, 4
10 ⁻⁴	0, 0, 0	2, 2	N.T.	N.T.
10 ⁻³	0, 0, 0	N.T.	"	"
10 ⁻²	3, 4, 0	"	"	"
10 ⁻¹	3, 3, 5	"	"	"

N.T. = not tested.

0 = guinea pig survived.

2, 3, 4 = guinea pig died in 2, 3, or 4 days after the inoculation.

Action of the Virus within the Eyeball

When a 10⁻¹ dilution of the fixed virus is injected into the eye, the animal invariably dies, usually from 72 to 96 hours after the injection. With this mode of inoculation it is easy to determine the minimum length of time the virus must act in order to produce a fatal infection. In a series of experiments the injected eyeball was surgically removed under deep ether anesthesia at different intervals following the inoculation. The eyeball can easily be removed intact, hemorrhage is negligible, and the animal always makes an uneventful recovery. A small quantity of pus may occasionally form in the eye socket but never causes symptoms of any sort.

The results of three such extirpation experiments following the injection of fixed virus are given in Table II. It is seen that when the

eye was removed in less than 6 hours, only 2 out of 17 animals died. When removal was performed 10 to 13 hours after inoculation, 8 out of 14 succumbed. All controls died. The period of 10 to 13 hours seems to be the average minimum time in which the virus must act within the eyeball in order to produce a fatal infection.

TABLE II

Guinea Pig Survival after Removal of Eyeball at Different Intervals after Injection with Fixed Strain of Virus

Experiment No.	Inoculated eye removed after					Controls
	1 hr.	3 hrs.	6 hrs.	10 hrs.	13 hrs.	
1	5, 0	0, 0*	0*, 0*	3, 3	N.T.	3, 3
2	0, 0*	0, 0, 0	4, 0, 0	3, 3, 0	3, 0	3, 6
3	N.T.	N.T.	0, 0, 0	3, 0, 0*	3, 3, 0, 0	3, 3

* In subsequent tests of immunity to intracerebral inoculation of 3×10^3 to 10^4 minimal lethal doses, the starred animals survived. Those not starred were not immune.

Other abbreviations as in Table I.

TABLE IIa

Identical Procedure with Unmodified Strain

Experiment No.	Eye removed after			Control
	$\frac{1}{2}$ hr.	1 hr.	3 hrs.	
1	6, 8	5, 0	4, 8	5, 0
2	5, 0	4, 5	5, 6	4, 0*
3	4, 0	5, 6	0, 0	4, 5, 5

* This animal showed marked signs of encephalitis, but recovered. Later histological examination showed healing encephalitis.

Survivors were not tested for immunity.

In the foregoing experiments the survivors were tested for immunity by the intracerebral injection of 0.1 cc. of a 10^{-3} dilution of virus, whose virulence by intracerebral tests in mice was constantly 10^{-6} or 10^{-7} . The animals that survived the immunity test are designated by a star in Table II. There is no constancy in the induction of immunity. One animal whose eye was removed 1 hour after inoculation was immune. Other animals, with removal after 13 hours, were not immune. The reasons for this variability are not clear.

Extirpation experiments were also carried out with the unmodified strain, as shown in Table II *a*. The fairly definite time interval required with the fixed strain is not necessary for the fresh virus. This property is undoubtedly correlated with the relative abilities of the two strains to invade the blood stream. The unmodified strain is readily found in the blood stream, but the fixed strain is recovered only rarely, or not at all (3).

Course of the Virus after Inoculation into the Eye

It was desirable to try to trace the course of the virus by examining different portions of the brain for virus content at different intervals of time after inoculation.

For this purpose 6 animals were inoculated into the right eye and sacrificed at different periods of time. The brains were removed aseptically and subdivided in accordance with the known optic pathways. The method of sectioning the brain was as follows: The cerebellum, medulla, and inferior colliculi were first cut off and discarded. With the brain ventral surface upward, gentle traction was applied to the optic chiasm, resulting in separation of the optic tracts from the underlying tissue. The chiasm and tracts were then cut away. The brain was turned dorsal side upward, and with small sharp scissors the lateral ventricle was entered from the medial surface of the hemisphere. The posterior neocortex of one side was then cut away, following the line of the rhinal fissure ventrally. The portion so removed included the entire area striata, and portions of the temporal, parietal, and occipital areas. It is impossible to differentiate these areas macroscopically. The procedure was repeated for the opposite side. Then the corpus callosum was cut through, and the two hippocampi and the hippocampal commissure gently peeled forward, exposing the thalamus and superior colliculi. The thalamus was separated from the hemispheres by incisions along the striae terminales. The thalamus and midbrain were then divided in the midline, and each half used separately. The entire olfactory portion of the brain, together with the basal ganglia and anterior neocortex, was left and utilized as a single portion. Throughout the dissection special care was taken to avoid contamination of one part by another.

This method of division gave 6 portions of the brain to be tested: the optic chiasm and tracts; the right thalamus plus superior colliculus; the left thalamus plus superior colliculus; the right and the left posterior neocortex; and the remainder of the hemispheres. The chiasm was ground up in 0.5 cc. of saline; the other portions were also ground in saline in approximately 10 per cent dilution. After light centrifugation, the supernatant fluid was injected intracerebrally into 3 to 4 week old mice, the dose being 0.05 cc.

The optic pathway was thus divided into three successive neurones: the optic chiasm and tracts, constituted by the axones of the ganglion cells of the retina; the lateral geniculate body and superior colliculus, where the optic tract terminates; and the visual cortex, which receives fibers from the lateral geniculate body. The thalamus and neocortex were tested bilaterally.

The results of this experiment are recorded in Table III. A minute trace of virus was detected in the contralateral geniculate body (and midbrain) 13 hours after the injection, although the optic chiasm did not show virus. At 24 hours there was abundant virus in the optic chiasm and the contralateral geniculate body and midbrain, but nowhere else. At 36 hours virus was present not only in these

TABLE III

*Spread of Fixed Virus after Inoculation into the Right Eye.
Virus Content of Brain Portions, Tested by Intracerebral Inoculation into Mice*

Guinea pig	Time of sacrifice	Optic chiasm	Left geniculate and midbrain	Left occipital cortex	Right geniculate and midbrain	Right occipital cortex	Remainder of brain
	<i>hrs.</i>						
1	10	0, 0, x	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
2	13	0, 0, 0	8, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
3	24	3, 3, 4	2, 3, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0, 0
4	36	2, 2, 3	2, 2, 3	2, 2, 0	0, 0, x	0, 0, 0	0, 0, 0
5	48	2, 2, 2	2, 2, 2	2, 2, 3	3, 4, 5	2, 4, 0	2, 4, 4

x = death occurred within a few hours of the inoculation, and was not due to virus action.

two regions, but also in the visual cortex of the contralateral side; other portions of the brain were still virus-free. At 48 hours, however, virus was widely disseminated throughout the entire brain.

The results of this experiment are entirely consistent with the view that the virus, inoculated into the eye, spread along the optic pathway to the thalamic centers (and midbrain), and from the thalamus progressed by the projection path to the cortex. These are the regions which first contain virus. The contralateral optic pathway was predominantly affected, since in the guinea pig the great majority of the optic fibers decussate.

To study further the time relationships involved, the foregoing

experiment was repeated, but only the optic chiasm and the left thalamus and midbrain were tested for virus content. Again inoculation was into the right eye. The results are given in Table IV. Again minute quantities of virus were detectable as early as 12 and 17 hours after inoculation, but significant quantities were first present at 20 to 24 hours. An important feature to be noted is the pres-

TABLE IV'

*Relation between Virus Content of Optic Chiasm and Tracts and of the Secondary Optic Centers, Following Inoculation into the Right Eye.
Tested by Intracerebral Inoculation into Mice*

Time	Experiment No.	Guinea pig	Optic chiasm	Left geniculate and midbrain
<i>hrs.</i>				
12	2	1	0, 0, 0	12, 0, 0
14	1	2	0, 0, 0	0, 0, 0
	2	3	0, 0, 0	0, 0, 0
		4	0, 0, 0	0, 0, 0
17	1	5	8, 0, 0	0, 0, 0
	2	6	0, 0, 0	0, 0, 0
		7	0, 0, 0	0, 0, 0
20	1	8	0, 0, 0	0, 0, 0
	2	9	2, 3, 0	0, 0, 0
		10	0, 0, 0	0, 0, 0
24	1	11	2, 2, x	2, 3, 4
	2	12	0, 0, 0	0, 0, 0
		13	2, 2, 0	0, 0, x

ence of virus in fairly large quantities in the optic chiasm and tracts before virus was apparent in the thalamus or midbrain (guinea pigs 9 and 13).

Pathology

The histopathology of the brain after infection with the fixed strain of virus has been found by Traub (3) to be substantially the same as with the unmodified virus. The first paper of this series (7) has

described in considerable detail the pathology of the natural virus disease in the guinea pig. Further study with the fixed strain reveals certain differences. After intracerebral inoculation the inflammatory reaction is vastly more pronounced, and the hippocampal degeneration is much more slight than after similar inoculation with the natural virus. The intensity of the inflammatory reaction is especially surprising in view of the considerably shorter duration of the disease (generally 2 to 3 days rather than 3 to 5).

In the present study, however, there are three principal points of interest in the pathology: the reaction occurring within the eye, the nature of the first lesions to appear within the brain, and the distribution of these lesions.

Intraocular Pathology.—Sections of eyes removed at different intervals, cut serially at 7 microns with short ribbons mounted every 50 sections, showed the development of the process. At about 13 hours there is a very slight reaction consisting of serum exudation, and a few mononuclear cells within the eyeball. In the connective tissue coats of the eye there may be numerous polymorphonuclear leucocytes. Within the eyeball, however, in uncomplicated cases, there is surprisingly little pathological change, even when the eye is removed at the death of the animal. A few leucocytes are present in the vitreous humor, and occasionally scattered leucocytes penetrate the outer layers of the retina. But there is no necrosis of tissue, and none of the focal reaction that is so prominent in the brain substance elsewhere following intraocular injection.

Sometimes there is a pronounced accumulation of polymorphonuclear leucocytes within the eyeball, and then the invasion of the surface of the retina by leucocytes is more intense, and necrotic ganglion cells may rarely be seen. This, however, is an entirely non-specific reaction. This histological picture has been duplicated by injection of normal brain emulsion diluted to the same degree as the virus suspensions used. Furthermore, when large numbers of leucocytes are present, intracellular bacteria may usually be seen in the exudate. Eyes that are sterile by bacteriologic culture always show an insignificant reaction to the virus (Figs. 1 and 2).

It may be mentioned that the slight degree of intraocular contamination sometimes becoming noticeable 24 or more hours after inoculation, has never had the slightest effect on the action of the virus.

Nature of the Cerebral Lesions.—In contrast to the lack of reaction in the eye, the histological picture in the higher optic centers is identical with that already described for the unmodified, natural virus. In 9 animals, sacrificed at intervals of from 24 to 52 hours, the entire thalamus and superior colliculus was sectioned serially at 10 microns, and every 15th section examined. In 5 other cases, from

48 to 65 hours, the entire brain was sectioned. Hematoxylin and eosin, phloxin and methylene blue, and thionin were the stains used.

The earliest change, first found at 24 hours, is a mild perivascular reaction, consisting of mobilized glial cells and mononuclear leucocytes (Fig. 3). This speedily develops into the typical inflammatory focus already described for the natural strain. As early as 36 hours after inoculation the typical focus may be very intense (Fig. 4). The earliest lesions are always found in the contralateral geniculate body or superior colliculus, followed 12 to 15 hours later by similar lesions in the same centers of the side of the inoculation. The series of cases studied, therefore, has double opportunities for observations of early lesions, first on the contralateral, then on the ipsilateral side.

Since the virus appears to spread along the nerve paths, it might be thought that a terminal nerve center might show neuronal necrosis as the first sign of disease. This is very definitely not the case. A closely graded series of cases supports the finding already published (7), that a vascular and interstitial reaction is the first sign of disturbance. These data further support the conclusion previously expressed (1), that there is no difference between lesions caused by blood-borne and those caused by nerve-borne virus.

The later lesions present no new features beyond what has already been described (7).

Distribution of Lesions.—The relatively crude method of gross dissection of the brain and testing of each portion for virus content, indicated that a spread of virus along the optic pathway was likely. The method of topographical analysis of the lesions (1) entirely supports the previous evidence. As early as 24 hours after right-sided injection there may be definite histopathology in the left superior colliculus or lateral geniculate body, rapidly growing more severe. The first lesions are sharply focal and rather rare, but they rapidly become more numerous and more intense until a maximum is reached at about 48 hours. Early lesions on the right side may be detected at 38 hours. At 48 hours the process has extended fairly widely into adjacent thalamic nuclei, so that large portions of this subcortical center are affected. The optic chiasm and optic tracts first show lesions at 36 to 38 hours, which is significantly longer than the time required to produce injury at the terminus of these tracts. The basal meninges begin to show inflammatory changes at about the same time as the optic tracts.

Pathologic changes in the left visual cortex are present to a very slight degree at 48 hours, but rapidly become more severe. At 53 to 55 hours there may be slight changes in the right posterior neocortex, but the temporal and occipital areas are more involved than the striate area.

At 48 to 53 hours there are numerous demonstrable lesions in areas which are functionally independent of the visual pathway. Such lesions may be present in various portions of the olfactory pathway, especially the tuberculum olfactorium and the amygdala, but also the septum; in the basal ganglia; in scattered unrelated areas of the anterior neocortex; in the hypothalamus, midbrain, and medulla.

DISCUSSION

Susceptibility to intraocular inoculation of fixed virus, as has been pointed out, lies intermediate between the intracerebral and the sub- or intracutaneous routes. The latter is fatal only with massive doses. The intracerebral route is always fatal in high dilutions. The intraocular route is fatal only in low dilutions (10^{-2} or 10^{-3}). The mechanisms involved appear to be somewhat different in the different methods.

The simplest explanation would seem to be as follows. Virus introduced into the eye infects the superficial ganglion cells of the retina. Once the cell body is infected, the entire neurone speedily becomes involved. That is, the virus then infects the cell processes making up the optic nerve. In the case of intraocular injection this takes place entirely within the central nervous system.

Such a spread of virus is quite different from that following a peripheral inoculation; with virus injected, say, into the thigh, any possible passage up the local nerve must infect the terminus of the nerve first, with subsequent passage toward the cell body. The entire metabolism of the neurone is controlled by the cell body. Passage of virus away from the cell body (centrifugal spread) seems to be in a different category from centripetal spread, at least, so far as this virus is concerned. The mechanism by which the virus, once it has infected the cell body, can "travel" along the axone, still remains completely unexplained. The multiplication of virus occurs with ex-

traordinary rapidity, and over a significant distance. Any attempted explanation at present would be sheer speculation.

The time interval of 10 to 13 hours required for virus to act within the eye is capable of different interpretations. This interval may represent the time required for the virus to work its way into the cell and begin its action. Or, it may include the time actually necessary for the virus to pass the length of the optic nerve and tract, and reach the geniculate body. There are no grounds as yet for a definite decision.

In Table II, Experiment 1, one guinea pig died although the eye was removed after an hour. This instance is similar to the behavior of the natural virus (Table II *a*) and may be an indication that in rare instances the fixed virus may act like the unmodified strain.

Some of the other animals whose eyes were removed and which yet survived, were immune to subsequent intracerebral inoculation. Undoubtedly a small amount of the virus escaped into the blood stream, not enough to produce fatal infection, but sufficient to immunize.

There are certain points of interest in the histological studies. Within the eye there is a negligible reaction although there the virus acts first and longest. On the other hand, elsewhere in the central nervous system the histological reaction is typical and in complete accordance with the descriptions previously given. This behavior of the eye appears strictly comparable to the behavior of brain tissue toward intracerebral inoculation. In the latter case, the site of injection of the virus shows merely a non-specific reaction to injury, although elsewhere in the brain there is well marked encephalitis. Furthermore, in another connection, guinea pigs have been infected by injection of virus into the cistern, so that the inoculum directly entered the cerebrospinal fluid. Here too the contact of virus and brain tissue caused only a mild and completely insignificant reaction. Characteristic pathology, found within the parenchyma, was not present at the surface of the brain in contact with the virus.

Experimental juxtaposition of virus and nerve tissue does not call forth the same reaction produced by the virus after its natural mode of spread. An analogy may be drawn with the data of vital staining

where trypan blue, for example, placed in direct contact with the brain, acts entirely differently from the same dye brought to the brain by the blood stream. These facts, and their relation to the "blood-brain barrier," are elsewhere discussed (6).

SUMMARY

The behavior of a fixed strain of Eastern equine encephalomyelitis virus was studied in guinea pigs after intraocular inoculation. Such inoculation concerns the central and not the peripheral nervous system.

The susceptibility to intraocular injection lies midway between the highly virulent intracerebral and the quite avirulent peripheral routes. The virus must act for 10 to 13 hours in order to induce a fatal infection. Removal of the inoculated eyeball before this interval almost always prevents fatality although it may allow immunity to develop. The virus, at suitable intervals after injection into the eye, may be recovered from successive and appropriate optic centers before it is demonstrable in non-optic portions. Approximately 24 hours are required for the virus to reach a significant concentration in the contralateral geniculate body, 36 hours in the contralateral visual cortex. Significant amounts of virus may be present in the optic chiasm and tract prior to involvement of the higher centers.

Virus placed in contact with the retina produces an insignificant, essentially non-specific reaction comparable to that produced at the site of direct intracerebral inoculation. In the retina there is no ganglion cell necrosis unless there is a complicating intraocular infection. In the cerebral visual centers the first reaction is inflammatory and interstitial, and may appear in the lateral geniculate body as early as 24 hours after injection. Neuronal necrosis is not the primary action of the virus on the nervous system in these experiments. The distribution of lesions in the brain is in excellent agreement with the method of direct testing for virus content, and is far more accurate than the latter.

The virus in its primary distribution through the nervous system follows the nerve pathways of the optic system. This occurs within the central nervous system, where presumably there is first an in-

volvement of the nerve cell body and then a spread along the cell process or axone.

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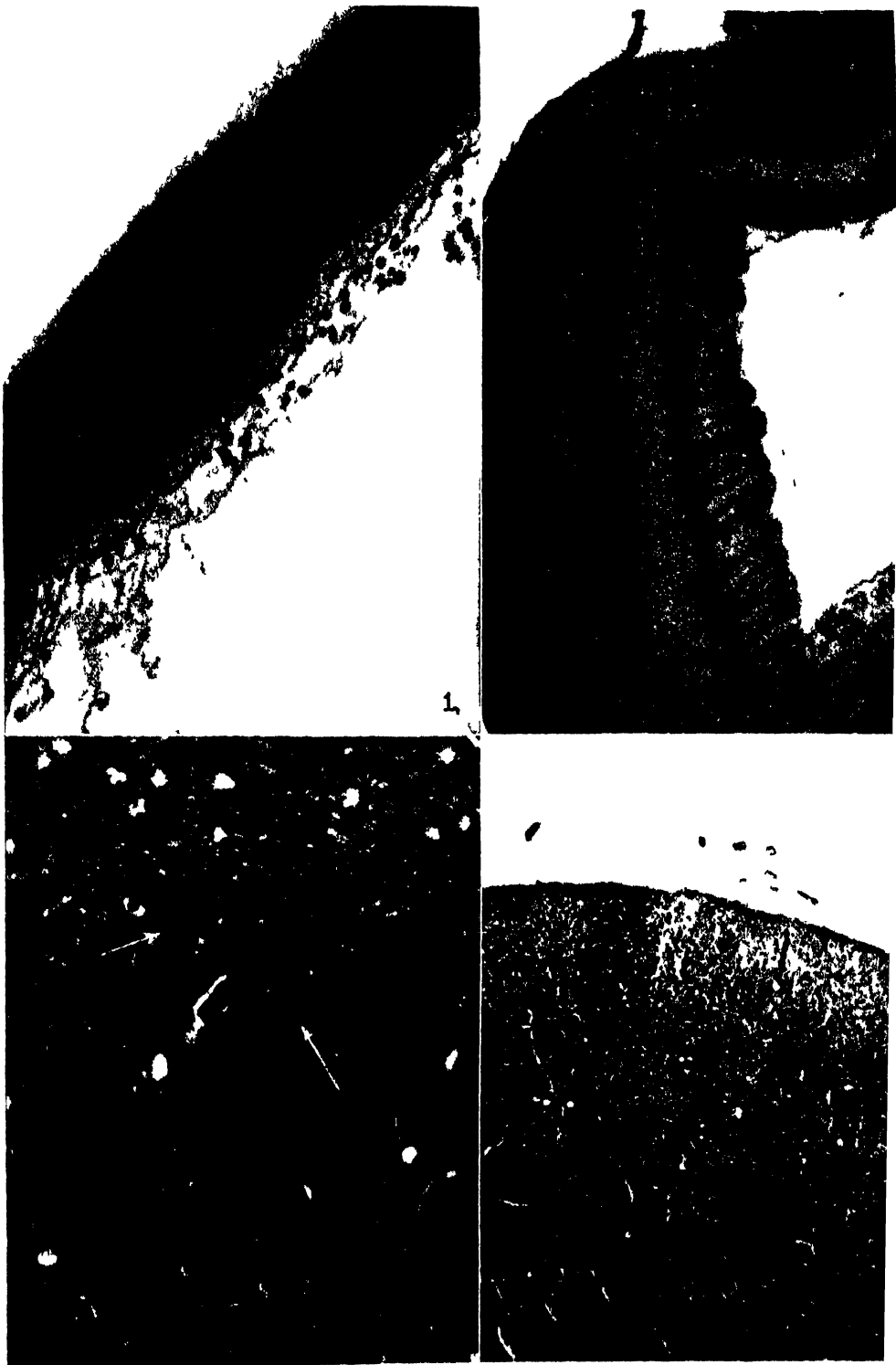
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EXPLANATION OF PLATE 41

FIGS. 1 and 2. Retina of injected eye of guinea pig. Eye removed at death, 66 hours after inoculation. In Fig. 1 are a few leucocytes on the surface of the retina; in Fig. 2, within its substance. The ganglion cells, as well as other elements, are intact. Phloxin-methylene blue. $\times 152$.

FIG. 3. Lateral geniculate body, contralateral to the injected eye. 24 hours after inoculation. The area indicated by the arrows shows a sparse infiltration with leucocytes and blood mononuclears, and some glial proliferation, all in relation to the blood vessel. There is no ganglion cell necrosis. Hematoxylin-eosin. $\times 152$.

FIG. 4. Superior colliculus, contralateral to the injected eye. 36 hours after inoculation. There is a very intense, circumscribed focus consisting chiefly of polymorphonuclear leucocytes. Apart from the inflammatory area, there is no neuronal necrosis. Hematoxylin-eosin. $\times 56$.



Photographed by J A Carlile

(King Eastern equine encephelomyelitis, 1

MOOSE ENCEPHALITIS*

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(Received for publication, March 13, 1939)

A disease of obscure nature affecting moose was referred to in the literature by Cameron and Fulton¹ in 1926, but practically all that is definitely known about the condition has been contributed by Fenstermacher and Jellison,² Fenstermacher,^{3, 4} and by Thomas and Cahn and coworkers.⁵⁻⁸

The diseased animals when first observed show but little of their customary fear of man. They may be fairly readily approached and may sometimes even be led into captivity. Weakness and unsteadiness, with a tendency to staggering gait but without true paralysis, are common. Less frequently there may be signs of impaired vision or peculiar attitudes of the head. Emaciation is observed in the majority of animals but this is by no means constant.

The condition does not show any definite seasonal incidence. Of 23 cases reported from Minnesota^{2, 3, 4} the distribution by months was as follows: April, 6; March, 4; October, 3; January, February and May, 2 each; June, July, September and December, 1 each. In Maine† the incidence of 20 cases was: March and April, 4 each; February, 3; May and June, 2 each; and January, July, August, October and December, 1 each. The majority of the cases have thus occurred in the months from February to May, but every month except November has had at least 1 case.

In pathological observations (sometimes incomplete) of 23 animals^{2, 3, 4} a variety of parasites was observed in the lungs, liver, intestine, heart and the eye in different animals. Most of the ani-

* An opportunity to study this disease was presented through the cooperation of Prof. E. C. Nelson of the University of Maine, and of Dr. F. Fenstermacher of the University of Minnesota.

† Personal communication from Prof. C. M. Aldous and Mr. A. L. Lamson.

mals showed infestation with the tick *Dermacentor albipictus*, frequently to an extremely severe and extensive degree. However, a rare animal may show no ticks at all and others show only minor degrees of infestation. Many of the moose showed a secondary type of anemia with basophilic stippling in the erythrocytes. In 2 cases evidence of inflammatory reaction was found in the brain. In 1 of these 2 animals an unidentified nematode was also found in the brain but without attending reaction. The inflammation occurring elsewhere in the brain was probably independent of this parasite. In 4 additional brains only small areas of hemorrhage were found. Apart from these observations in the brain, pathological features that might be relevant to the disease picture were not noted.

Attempts at autopsy to recover pathogenic infectious agents were uniformly unsuccessful, not only by ordinary bacteriological culture, but also by intracerebral or other inoculation of tissue suspensions, including that of the brain.^{2, 3, 4} On the other hand, Thomas and Cahn and associates⁵⁻⁸ studied ticks infesting pieces of moose hide cut from dead animals and shipped to Illinois. When guinea pigs were infested with these insects a fatal disease was produced supposedly similar to what had been described in the moose. From the ticks a new bacterium was isolated, *Klebsiella paralytica*, whose properties have been carefully described⁸ and which was suggested as the cause of the disease in moose. However, Fenstermacher, working directly with moose as soon as they were killed, has been unable to confirm these observations. A bacterial origin for "moose disease" cannot be considered as established.

A typical example of the disease was observed at the University of Maine, with the following history.*

On October 21, 1938, a moose was reported wandering around on the highways by the wardens near West Rockport, Maine. It seemed fairly strong but was thin and very tame. It had been seen for 3 days when reported. The animal was loaded into a truck and brought to Orono and placed in about an acre sized pen in the woods. In the pen it showed a good appetite and browsed; it was

* Gathered by Mr. Lamson and Dr. Witte, and kindly supplied to the author by Prof. E. C. Nelson.

also fed carrots, cod liver oil and bone meal. On November 10th marked drowsiness, drooping of the hind quarters and a tendency to fall developed. On November 13th the animal fell in a brush pile and was able to get up only with help. A peculiar lopping and a flick of the right ear were noticed, as well as an intermittent facial twitch on the right side. On November 14th the animal went down and could not get up even with help. A pronounced right twist of the neck was noted the next day. On November 16th the moose lay flat on its left side with the neck extended. Marked edema of the eyelids was present and the eye on the left side was clouded and apparently blind. The animal was shot through the heart and autopsied immediately.

The head was removed at autopsy, packed in solid carbon dioxide and shipped to this laboratory, where it arrived on the 2nd day following. The tissues were in an excellent state of preservation, considering the lapse of time since death. On removal of the brain no gross abnormalities were observed. The nose, sinuses and ears were entirely normal. The edema of the eyelids mentioned in the history was not observable after the lapse of time involved during shipment, and the eyeballs showed no abnormalities. Some of the brain material was saved for passage and the remainder fixed in 10 per cent formalin or in Zenker's fluid. This animal is designated hereafter as Moose 1.

For further study the following additional material was obtained from Prof. Nelson: portions of a brain and cord of a 2nd moose fixed in 10 per cent formalin for 11 months, and an entire brain of a 3rd animal, fixed in formalin for 8 months. Dr. Fenstermacher kindly furnished paraffin blocks of parts of the brain from 5 additional animals which had been autopsied in Minnesota. Material from 8 animals was thus studied.

Observations

The following description is drawn chiefly from Moose 1 whose history has been detailed above. Reference to the other cases is made where indicated.

In the brain several different types of pathological change may be observed, the interrelations of which it is not always easy to

determine. There is a definite loss of myelin, although of an unusual type. In frozen sections stained with scarlet red considerable quantities of neutral fat may be observed in scattered parts of the white matter. In Figure 1 is seen a low power view of a portion of the corpus callosum illustrating an unusually severe degree of fat formation. The fat stains a brick red, rather dull in color, without the brilliance frequently seen in other demyelinating conditions. Under polarized light there is no double refraction. The lipid droplets do not stain with hematoxylin in myelin stains. That all the fat is intracellular, within phagocytes, cannot be satisfactorily shown. Stains of cells in paraffin embedded tissues show very few typical compound granular corpuscles. This may be due to cytoplasmic disintegration in the period between death and fixation of tissue, or it may be due to the absence of such cells. That neutral fat can occur in the brain in the absence of phagocytic or other cellular action is not surprising, for this type of fat formation has previously been demonstrated in multiple sclerosis.⁹ Similar, though less intense areas of fat formation have been found in the centrum ovale and in the white matter of the cerebral convolutions. In such regions, as in Figure 1, the lipid droplets are scattered more or less uniformly in very poorly defined foci. Sharply circumscribed areas showing loss of myelin, such as are seen in multiple sclerosis, do not occur. Where the free fat is scattered in the tissue the myelin sheaths may be moderately diminished in number but are not totally lost.

The presence of diffusely scattered fat is indicative of an acute process. In other portions of the brain there is an accumulation of lipid only in the perivascular spaces, but none in the intervascular areas. Such a field is illustrated in Figure 2 and is a sign of an older process than that shown in Figure 1. The total amount of fat observed in the entire brain, both in the parenchyma and in the perivascular spaces, is not large.

In other regions, where there is no sign of recent destruction, many scarred areas demonstrable with stains for glial fibrils are found. These scars are present in the medulla, centrum ovale and convolutional white matter of the cerebrum, and also in the white matter of the cerebellum. In Figure 3 is seen such a scar in the medulla. The proliferation of glial fibrils is quite intense but the

actual increase in astrocytes, *i.e.*, in the number of cell bodies, is not great. The occurrence of intense fiber proliferation, in the absence of significant cellular increase or of progressively altered cell forms, shows that the repair process is completed. The original insult occurred probably some months previously.

Two types of glial scars are illustrated in Figures 4 and 5. Figure 4 is from the centrum ovale of Moose 1 and shows several small perivascular scars. Figure 5, from the cerebellum of Moose 6, shows a dense isomorphous glial feltwork, diffuse rather than perivascular. Here the process is quite old, for well defined astrocytes are rare, although the fiber proliferation is great. On the other hand, in Figure 6, from Moose 1, astroblastic forms are clearly seen among the glial fibers.

The loss of myelin is generally mild, invariably perivascular, and practically always much less in extent than is the fibrous gliosis. In Figure 7, from Moose 1, the destruction of myelin, although slight in absolute terms, is very severe as compared with that in other parts of the brain. In Figure 8 is seen a section, adjacent to that shown in Figure 3, of the medullary reticular formation but stained for myelin. Considering the thinness of the section (12μ) it is readily seen that the loss of myelin is disproportionately small compared with the density of the gliosis (Fig. 3). And in the myelin stained section adjacent to that of Figure 5 no loss of myelin at all can be discerned in the corresponding area. In the section adjacent to that of Figure 4, but appropriately stained for myelin, there are small clear areas surrounding the affected blood vessels.

Axis cylinders are somewhat better preserved than the myelin sheaths but not to any marked degree. But, as has been emphasized elsewhere,¹⁰ in the loss of myelin from whatever cause the axis cylinders are always less affected than the myelin sheaths.

There is abundant cellular reaction, chiefly perivascular. In Figure 2, for example, the cells in the blood vessel sheaths are chiefly concerned with the phagocytosis of lipoids. Elsewhere, however, as shown in Figure 9, there may be a true inflammatory reaction with abundance of lymphocytes around the blood vessels and even some diffuse tissue infiltration. Polymorphonuclear leukocytes were not seen. This inflammation may be of the "secondary" or symp-

tomatic type for, as is well known, it is frequently seen in various non-infectious demyelinating diseases. In the sections corresponding to Figure 9, but stained for glia and myelin, a fibrous gliosis could not be demonstrated. Around the more severely involved blood vessels was a mild degree of loss of myelin.

With one single exception, the inflammatory reaction, where present, was restricted to the white matter. In the exceptional instance (Moose 5 of this series) the gray matter was affected. This occurred in 1 case sent by Dr. Fenstermacher where, slightly involving the entorhinal cortex, there was a reaction very similar to what he has illustrated in his Figure 3.³ Moose 5 of this series is the same animal from which Dr. Fenstermacher's photograph was taken.

The neocortex was intact in all available material. For the most part the tissue was not sufficiently well preserved for cytological examination. Most of the ganglion cells showed severe swelling, vacuolation, and other postmortem artefacts. But the architectonics appeared normal and no areas of loss of cells, inflammation, or of meningitis were observed.

Of the 8 cases available for study, 3 came from Maine, of which 2 showed similar pathological lesions, while in the 3rd no lesions of any sort could be found. This last case, however, stained poorly. Of the 5 cases, blocks from which were sent from Minnesota by Dr. Fenstermacher, gliosis with more or less demyelination was found in 2. In a 3rd, of which only a few blocks were available, the only pathological change observed was the inflammatory reaction in the gray matter referred to above. In the remaining 2 cases no abnormalities could be detected. In 1 of these 2, however, death was probably due to distomiasis and not to moose encephalitis (personal communication from Dr. Fenstermacher; material to be published). Thus, in 7 probable cases of the disease in question, characteristic changes were observed in 4, while 1 other appeared atypical. In reference to the negative findings it should be pointed out that all sick (or dead) moose, even with fairly similar symptoms, are not necessarily affected by a single disease. Fenstermacher² is strongly of the opinion that "the losses of moose that occur in Minnesota are not the result of a single pathogen."

From the whole brain that was received unfixed from Maine, portions were emulsified for animal passage. Sheep, kittens, mice and a pig were inoculated. One sheep died of bacterial meningitis, but a 2nd animal survived without symptoms. All the other injected animals also showed no symptoms. These results agree with Fenstermacher's inability to reproduce the disease by inoculation.

DISCUSSION

The occurrence of neutral fat, perivascular areas of demyelination, gliosis, and moderate inflammatory reaction in the brains of moose raises the question of a possible relation to multiple sclerosis and other demyelinating diseases of man and animals. One chief difference from multiple sclerosis is that in the latter disease the areas showing loss of myelin, although frequently perivascular in early lesions, usually develop to have no relation to blood vessels.¹¹

In the moose, the loss of myelin is disproportionately small compared with the extent of the gliosis, somewhat reminiscent of the human cases reported by Müller¹² and by Bodechtel and Guttmann,^{13, 14} and not at all similar to multiple sclerosis. It is necessary to agree with their statement that gliosis is not merely a defect filler but may be induced independently. In a previous communication⁹ it was pointed out that the gliosis which occurs in multiple sclerosis cannot be considered as merely secondary to the loss of myelin. This statement must be repeated in relation to the disease in moose.

It is well known that in the central nervous system there is no necessary connection between inflammation and loss of myelin. Glial proliferation may occur as a result of an inflammatory reaction in which myelin has not been significantly destroyed. Yet in the disease in moose the histological picture does not suggest a primary inflammatory condition such as is found in many virus diseases. An exception to this statement is Moose 5, referred to above, with a typical primary inflammatory reaction involving the gray matter, a condition which does not fit in with other cases of the series. Although in this single case only a few blocks were available for examination, the evidence is strongly suggestive that this one instance may represent a quite different disease entity. In the 4 other positive

cases the changes observed were strictly those of a leukoencephalitis. Moose 5 was not in this category.

For the present, until further data become available, this leukoencephalitis must be considered as a disease entity in moose, with the strong possibility that there may also be another form of encephalitis.

It must be emphasized that the disease process as disclosed by the present study is evidently a subacute or chronic condition. The glial scars are at least of several months duration, and not improbably even older. At the same time activity of the disease shortly before death is shown by the occurrence of neutral fat. The immediate cause of death, however, as in most neurological conditions, is not apparent.

The etiology of the demyelinating condition remains obscure. Attempts by Fenstermacher and by ourselves to transmit the condition by tissue inoculation have been negative. This failure is not a cogent argument against an infectious etiology, but the evidence is supported by the fact that none of the many forms of leukoencephalitis has ever been shown to be caused by an infectious agent. That the bacterium *Klebsiella paralytica* is the causative agent must remain questionable until confirmed. Attempted confirmation has not proved successful.³

The rôle of the tick infestation remains equally obscure. The condition of tick paralysis, in animals and man, is well recognized as a naturally occurring disease and has been reproduced experimentally.^{15,16} However, there are no adequate studies of the pathology of this condition, and the clinical course does not suggest a kinship. A toxic factor resulting from the tick infestation cannot be arbitrarily ruled out.

Thrombosis and vascular occlusions as the cause of demyelinating lesions have been claimed by Putnam.¹⁷ In the present instances evidence of thrombosis was not observed.

There is no evidence throwing satisfactory light on the etiology of this disease. Assuming that the great majority of dead or sick moose observed are suffering from a single disease entity, and considering the total moose population of Maine and Minnesota, the incidence of this disease is high, suggesting either an infection or a

common environmental factor such as a dietary deficiency or a toxic substance. Grounds for a decision are not as yet available.

SUMMARY

A subacute or chronic leukoencephalitis occurring naturally in moose is described. The characteristic picture consists of a mild degree of perivascular demyelination, with formation of neutral fat, and with fibrous gliosis disproportionate in extent to the loss of myelin. There may be mild inflammation restricted to the white matter. There is suggestive evidence that a primary inflammatory reaction involving gray matter and observed in 1 animal out of 8 may represent a separate condition. Attempted animal passage of fresh material from 1 case was unsuccessful. The etiology of this leukoencephalitis is obscure although various possibilities are discussed.

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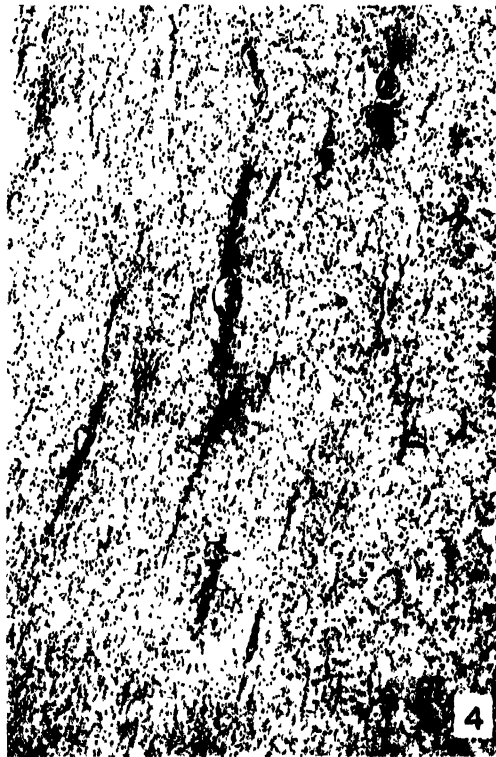
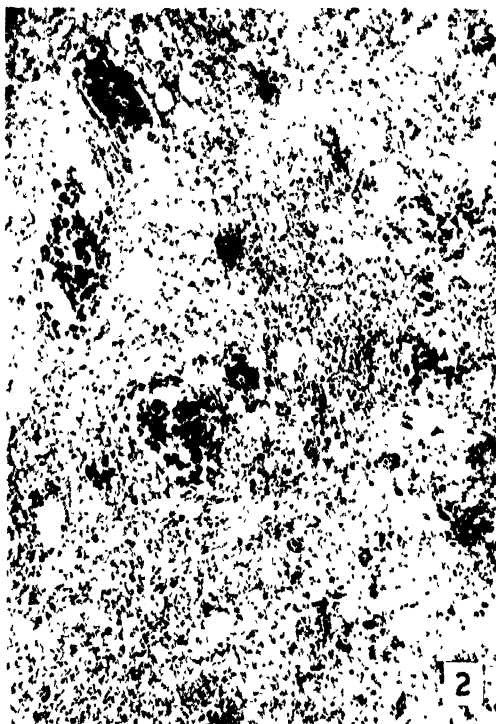
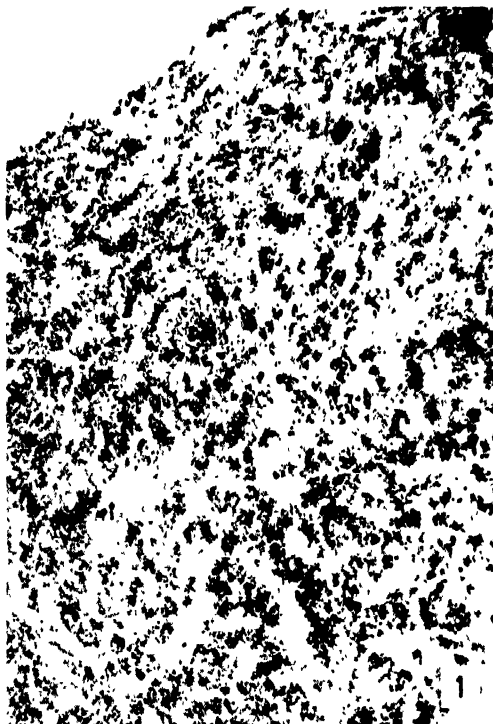
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DESCRIPTION OF PLATES

PLATE 74

- FIG. 1. Cerebral white matter, stained for fat. The stainable lipoids are diffusely scattered throughout the affected focus. $\times 50$.
- FIG. 2. A different field from the same animal. The neutral fat is located almost exclusively in phagocytes in the perivascular spaces. The insignificant loss of myelin can be readily appreciated even with the fat stain. $\times 133$.
- FIG. 3. Reticular formation of the medulla oblongata, stained for glial fibers with Victoria blue. Dense, moderately well circumscribed perivascular gliosis is present. This figure should be compared with Fig. 8, the same field of an adjacent section stained for myelin. $\times 105$.
- FIG. 4. Centrum ovale, stained for glial fibers. The small scars are strictly perivascular. $\times 47.5$.



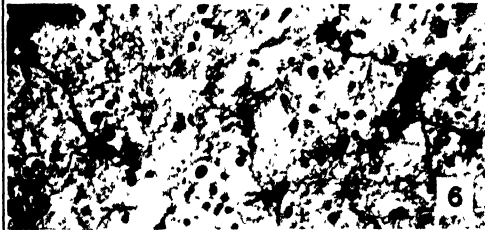
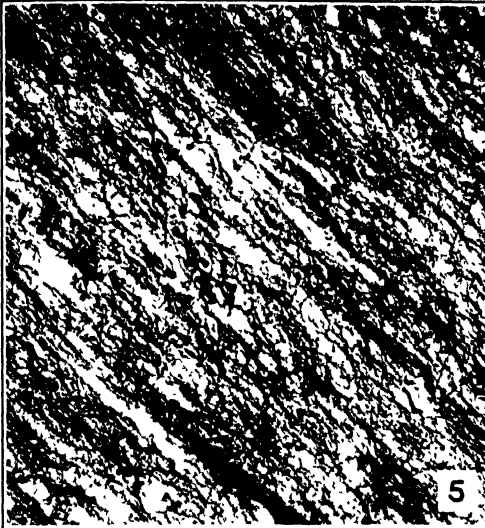
King

Moose Encephalitis

MOOSE ENCEPHALITIS

PLATE 75

- FIG. 5. Cerebellar white matter, stained for glial fibers. There is a very dense glial feltwork. Adjacent sections stained for myelin show no loss of myelin. $\times 180$.
- FIG. 6. From the centrum ovale, showing astroblastic forms in the midst of a mild diffuse gliosis. $\times 230$.
- FIG. 7. Cerebral white matter, stained for myelin. A slight degree of demyelination is evident around the smaller blood vessels. $\times 18.6$.
- FIG. 8. Reticular formation of medulla oblongata, stained for myelin. Fig. 3 shows the dense glial scar in this region. Fig. 8 illustrates the insignificant degree of loss of myelin. $\times 105$.
- FIG. 9. Centrum ovale. Inflammatory reaction in the white matter, not invading the cortex, visible at upper left. Toluidine blue stain. $\times 35$.



DISSEMINATED ENCEPHALOMYELITIS OF THE DOG

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In 1930 Perdrau and Pugh,¹ in England, called attention to demyelinating lesions in the central nervous system of the dog suffering from what they called "the 'nervous form of canine distemper.' " They found that 4 of 14 animals showed this reaction. The demyelination, when present, was observed chiefly in the cerebellar peduncles or folia and was generally accompanied by heavily cuffed blood vessels.

At the same time, but independently, Posrednik² described similar lesions. In 9 cases of experimentally induced distemper with a greater or a less degree of encephalitis, he observed a very slight degree of demyelination in 1 instance. But in 3 cases of the disease occurring naturally the loss of myelin was more pronounced, especially in the cerebellum, medulla and pons. Subsequently Marinesco, Draganesco and Stroesco,³ as well as Peters and Yamagiwa,⁴ gave detailed descriptions of the histologic changes in cases of distemper and confirmed the observation of loss of myelin in many of the lesions.

The literature on distemper is extensive. This virus-induced disease may attack the nervous system to produce nervous manifestations but does so inconstantly. Apart from the loss of myelin which is sometimes observed, the principal features described by the older investigators are those of encephalitis and meningoencephalitis, namely: perivascular accumulation of lymphocytes; inflammatory infiltrations involving the parenchyma and the meninges, sometimes very intensive; occasional perivascular hemorrhage; endothelial pro-

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liferation; variable degrees of degenerative neuronal damage; changes in the glial apparatus, both proliferative and regressive. The intracellular inclusions, first described thirty years ago, have recently been studied and the relevant literature reviewed by De Monbreun.⁵ He found the inclusions abundantly present in the brain as well as in other diseased tissues. Many authors, however, have not been able to find inclusions in their cases, although, among recent writers on the neuropathology of the disease, excellent illustrations have been given by Gallego⁶ and by Marinesco, Draganesco and Stroesco,⁷ as well as by De Monbreun.⁵

The observations mentioned are those which occur when the animal shows clinically involvement of the nervous system. But Cerletti⁷ described a series of dogs suffering from clinical distemper without nervous manifestations in which, nevertheless, inflammatory and toxi-degenerative lesions of the nervous system were present. This observation has been confirmed by Roman and Lapp.⁸

Although the foreign literature contains numerous studies on disseminated encephalomyelitis of the dog and its relation to distemper, little attention has been paid to the subject in this country. No observations have been made on the problem of demyelination. The following report of a case is consequently of some neuropathologic interest.

CLINICAL HISTORY

The dog reported on here was sent to this laboratory by Dr. F. A. Zucker, of Roselle, N. J., to whom I wish to express my thanks for his cooperation.

The available history is rather sketchy. The animal was a pointer bitch, about 4 years old, first taken ill about three months previously. The original symptoms are not on record, but the animal was first treated by a veterinarian for tapeworm. A second veterinarian diagnosed brain fever. When seen by Dr. Zucker, the animal showed spasticity, stiffness of gait, with clonic movements of the forelegs, ataxia and mental impairment. The pupils did not react to light, and there was no response to pinprick over the posterior portion of the body. There was slight improvement for a few days, but then the animal became rapidly worse and was sent to this laboratory for examination.

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7. Cerletti, U.: *Ztschr. f. d. ges. Neurol. u. Psychiat.* **9**:520, 1912.

8. Roman, B., and Lapp, C. M.: *Bull. Buffalo Gen. Hosp.* **3**:40, 1925.

When seen by me, the animal was in a recumbent position, lying on the right side, in marked distress. There were marked decubitus ulcers on the right shoulder and elbow. The hindlegs were flexed and completely paralyzed. Moderate contractures were present. The right foreleg was in rigid extension and strongly resistant to any passive movement. The left foreleg was only moderately spastic. No tendon reflexes were elicitable. The sensory deficit was marked, although no sharp level could be detected. Pinching and mildly painful stimuli applied to the hindlegs and trunk evoked no response. Some sensation apparently remained in the forelegs and head. Pupillary and corneal reflexes were present.

The animal was killed by chloroform anesthesia. During the struggle only the left foreleg and the head were spontaneously moved. During narcosis the spastic right foreleg became flaccid. The hindlegs showed only moderate relaxation due to the contractures.

The general autopsy showed only severe hemorrhagic cystitis. The bladder was not dilated, but the wall was markedly thickened, and the mucosa showed inflammation, hemorrhage and necrosis. There were no other features of significance. The involvement of the bladder was undoubtedly secondary to the injury of the spinal cord.

With regard to the nervous system, in gross the most marked changes were in the spinal cord, which in portions was very soft. On section cavities were visible in some regions around the central canal. The brain showed no gross abnormalities.

The brain and cord were fixed in 10 per cent formaldehyde; pyroxylin, paraffin and frozen sections were made of representative regions.^{8a} Hematoxylin-eosin, thionine, iron-hematoxylin, Van Gieson, scarlet red, Weil, silver carbonate, Bodian^{8b} and Anderson^{8c} staining methods were used.

MICROSCOPIC OBSERVATIONS

The changes in the nervous system are primarily inflammatory, although varying in intensity. The diseased areas are scattered throughout the entire neuraxis, with the greatest intensity in the spinal cord. The lesions in the cord are not confluent but scattered throughout its length. Some lesions, however, extend over several segments.

Spinal Cord. A typical lesion is a fairly well delimited focus occurring in either the gray or the white substance but with greater frequency in the latter. Figure 1 *A* illustrates such a lesion. The

8a. The Weil staining method is described in McClung, C. E.: *Handbook of Microscopical Technique*, New York, Paul B. Hoeber, Inc., 1937, pp. 474.

8b. Bodian, D.: *Anat. Rec.* **65**:89, 1936.

8c. Anderson, J.: *How to Stain the Nervous System*, Edinburgh, E. & S. Livingstone, 1929, p. 80.



FIG. 1.—*A*, spinal cord, anterior-lateral portion. There are moderately intense perivascular infiltration and proliferation of vascular endothelium. The cribrated appearance of the white matter should be noted. On the extreme lower right, the myelin is essentially normal. Hematoxylin-eosin stain; $\times 61.5$. *B*, spinal cord, showing infiltration of the parenchyma with plasma cells and occasional polymorphonuclear leukocytes. Hematoxylin-eosin stain; $\times 431.2$.

blood vessels stand out because of the perivascular infiltration by lymphocytes, plasma cells and more undifferentiated mononuclear cells. Under higher power the endothelium may, in places, be seen to be proliferated. Between and around the blood vessels the tissue is rarefied, presenting a lacunar appearance, but axis-cylinders persist in large numbers. Apart from the sheaths of blood vessels there is little or no increase in cellularity in the damaged area. Although some progressively altered glial forms may be observed, there are no gitter cells in this particular lesion.

There are several variations on this picture, each of which presents its own significant features. Two fields from a single section illustrate different pathologic processes. In figure 1 *B* there is shown a very intense cellular reaction. The larger blood vessels (outside the field of this photomicrograph) are very heavily cuffed, but in the particular field shown the infiltration of the tissue by plasma cells is noteworthy. Gitter cells are present in moderate numbers, as well as other microglial forms. Occasional polymorphonuclear leukocytes are also scattered through the tissue. The lacunae in the tissue may, to a certain extent, be seen in process of formation, but the cellular infiltration is so heavy that details of the tissue are obscured. This is considered to be an acute stage of the process shown in figure 1 *A*. On the other hand, elsewhere the *état criblé* may be very marked, even more than in figure 1 *A*, and yet the interstitial reactions may be entirely negligible.

A quite different picture is shown in figure 2 *A*. Here there is practically complete softening, involving the anterior horn and adjacent white matter. The reactive cells are almost exclusively compound granular corpuscles, and these are not numerous. Most unusual, however, is the excellent state of preservation of the anterior horn cells in the midst of the tissue *débris*. The nuclear membranes are hyperchromatic, but the general cellular appearance is excellent. There are a few neurons that show degenerative changes, but they are exceptional. However, in other areas, in other sections of the cord, a similar type of damage of the tissue has destroyed the neurons as well.

Sections of the spinal cord stained for myelin show expected changes. Figure 2 *B*, of the cervical region, displays different degrees of demyelination, in accordance with the cellular picture. The regions of

spongy vacuolation and rarefaction, with moderate perivascular reaction, show considerable diminution in the quantity of myelin but not total loss. Other areas show practically complete destruction of myelin. In the center there is cavitation, due to complete softening. Many of the gitter cells contain hematoxylin-staining masses of myelin, testifying to the acuteness of the process. Longitudinal sections of the cord (fig. 2 C) show an irregular margin as well as the usual beadings and swellings attendant on myelin disintegration.

An instructive field is illustrated in figure 2 D, taken from the lateral column of a cross section of the thoracic portion of the cord. Numerous punched-out areas of demyelination are present in otherwise well preserved tissue. Closer examination shows that all of these areas are heavily cuffed blood vessels, where the perivascular accumulation of cells has choked out the myelin. Some of the inflammatory cells contain minute droplets of myelin. The intervening tissue appears relatively normal. This type of demyelination seems to be in a different category from that previously illustrated. It is more comparable to the strictly focal demyelination sometimes seen after hemorrhagic encephalitis⁹ rather than to the diffuse damage of tissue seen in figure 1 and figures 2 A, B and C. It is very doubtful that this perivascular type would develop into the diffuse forms, in which the tissue as a whole appears injured. The pathogenic factors in the two forms appear to be distinct.

Sections of the spinal cord stained for fat confirm the observations on the sections stained with hematoxylin and eosin. The amount of neutral fat, although in some areas fairly considerable, is on the whole much less than would be expected, certainly incomparably less than would be seen in so-called acute multiple sclerosis of comparable duration. The gitter cells, as has already been noticed, are not very numerous except in certain areas of almost total softening. The Herxheimer stain shows many cells, both in the perivascular sheaths and free in the tissue, which contain a few small globules of neutral fat. The heavily crammed compound granular corpuscles are present in certain areas, but where the infiltration of the tissue is heaviest they are few.

9. Baker, A. B.: *Am. J. Path.* **11**:185, 1935. Russell, D. S.: *J. Path. & Bact.* **45**:357, 1937. Dobbs, R. H., and de Saram, G. S. W.: *ibid.* **46**:437, 1938.

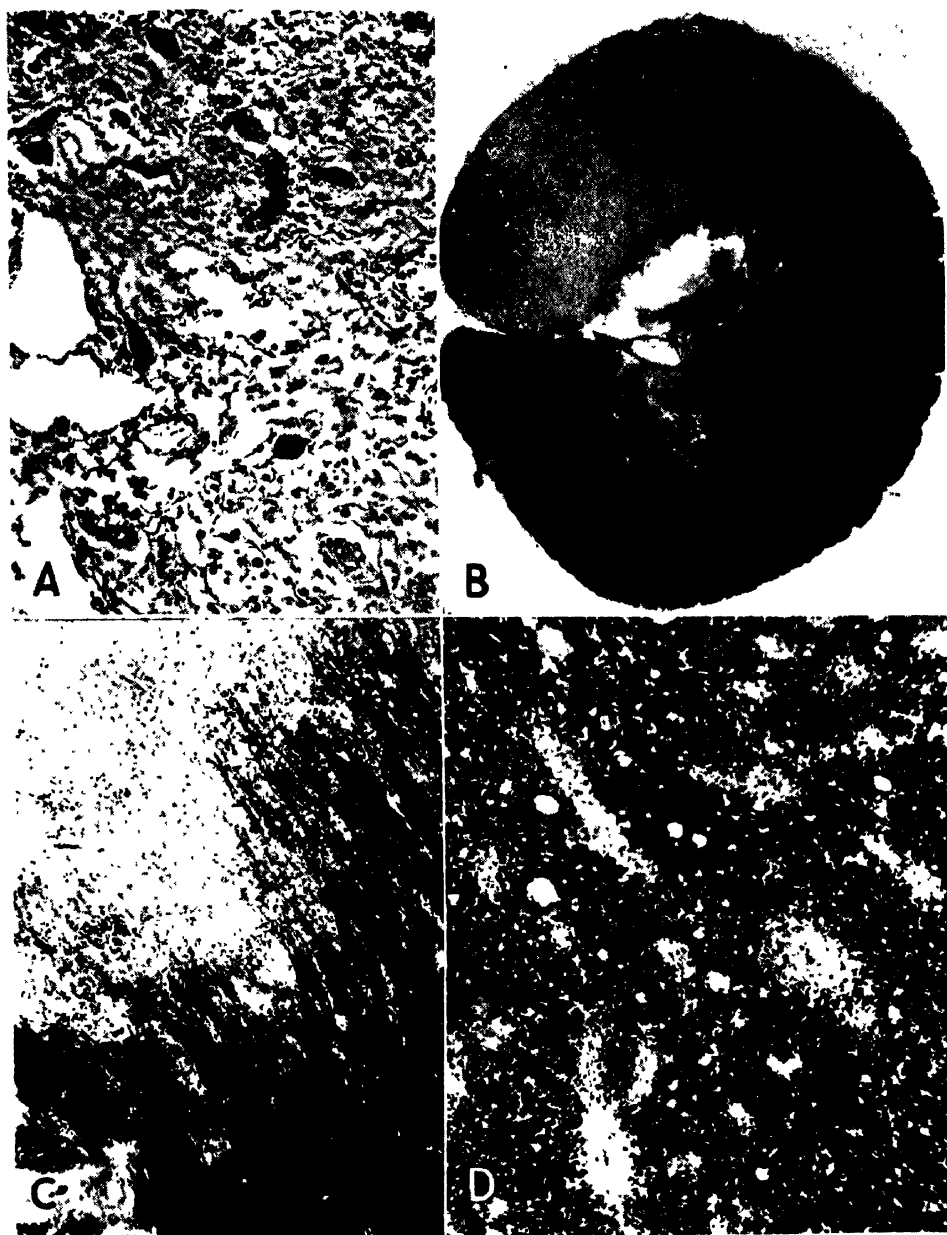


FIG. 2.—*A*, spinal cord. There is almost complete softening of part of the anterior horn and adjacent white matter. The reactive cells are almost exclusively compound granular corpuscles. The excellent preservation of the ganglion cells in the midst of the tissue débris should be noted. Hematoxylin-eosin stain; $\times 85.8$. *B*, spinal cord, showing partial and complete demyelination. The état criblé is readily apparent. Weil stain; $\times 9.4$. *C*, spinal cord, longitudinal section, illustrating the sharp margin of the demyelinating process. Much hematoxylin-staining material is still present in the demyelinated area. Weil stain; $\times 34.5$. *D*, transverse section of spinal cord, showing perivascular demyelination consequent to intense perivascular infiltration. There is much phagocytosed myelin in the perivascular spaces. Weil stain; $\times 72.7$.

The axis-cylinders are invariably better preserved than the myelin. Even where the destruction of tissue appears most complete, axis-cylinders persist, though distorted and damaged. In other areas they are preserved in considerable numbers. All types of degenerative changes, such as varicosities, torpedoes, swellings, splittings, fibrillary ball formations, loops and the like, are abundantly in evidence.

Repair has taken place to a certain extent, but instead of gliosis (i.e., proliferation of fibrous astrocytes and their fibers) chiefly the connective tissue is involved. Figure 3 *A* illustrates a rather marked degree of this process. For comparison with the connective tissue reaction in multiple and diffuse sclerosis, reference may be made to a previous paper.¹⁰

Medulla and Higher Levels. The pathologic changes differ from those of the preceding description chiefly in degree. In general they are not so severe as those in the spinal cord.

In the medulla and pons there are scattered lesions involving the cerebellar peduncles, various scattered areas at the periphery and less frequent areas in the more central portions. Figure 3 *B* illustrates the degree of damage in the pons. In general there is fairly heavy perivascular cuffing with plasma cells and lymphocytes. Rarefaction of tissue and lacunar degeneration are common, but white blood cells are not frequent in the parenchyma. Numerous compound granular corpuscles are present. Apart from the discrete lesions the tissue is entirely normal.

In the cerebellum too the process is similar. The damaged areas are fairly well circumscribed and scattered through the cerebellar white matter. The perivascular infiltration is often quite dense, and infiltration of the tissue by inflammatory cells may be well marked. However, it is nowhere as severe as in the spinal cord. In the cerebellum itself, in contrast to the cerebellar peduncles, medulla and cord, there is much less of the *état criblé*. The demyelination is more complete and not attended by lacunar degeneration of the tissue. Figure 3 *C* illustrates lesions in the cerebellar white matter.

Areas may be seen similar to those in figure 2 *D*, i.e., small "holes" in the myelin caused by the heavily cuffed blood vessels. The inter-

10. King, L. S.: Arch. Path. **23**:338, 1937.



FIG. 3.—*A*, reticulin proliferation in a diseased area of the spinal cord. Silver carbonate impregnation for connective tissue; $\times 71.6$. *B*, brain stem cut through the inferior colliculi and pons, showing demyelination in the pons. Arrows point to less obvious areas. Weil stain; $\times 3.6$. *C*, demyelination in the cerebellum. Arrows point to early foci. Weil stain; $\times 4.25$. *D*, inflammatory focus in the caudate nucleus, bordering the ventricle. Foci in the cerebral cortex are very similar. Hematoxylin-Van Gieson stain; $\times 30.3$.

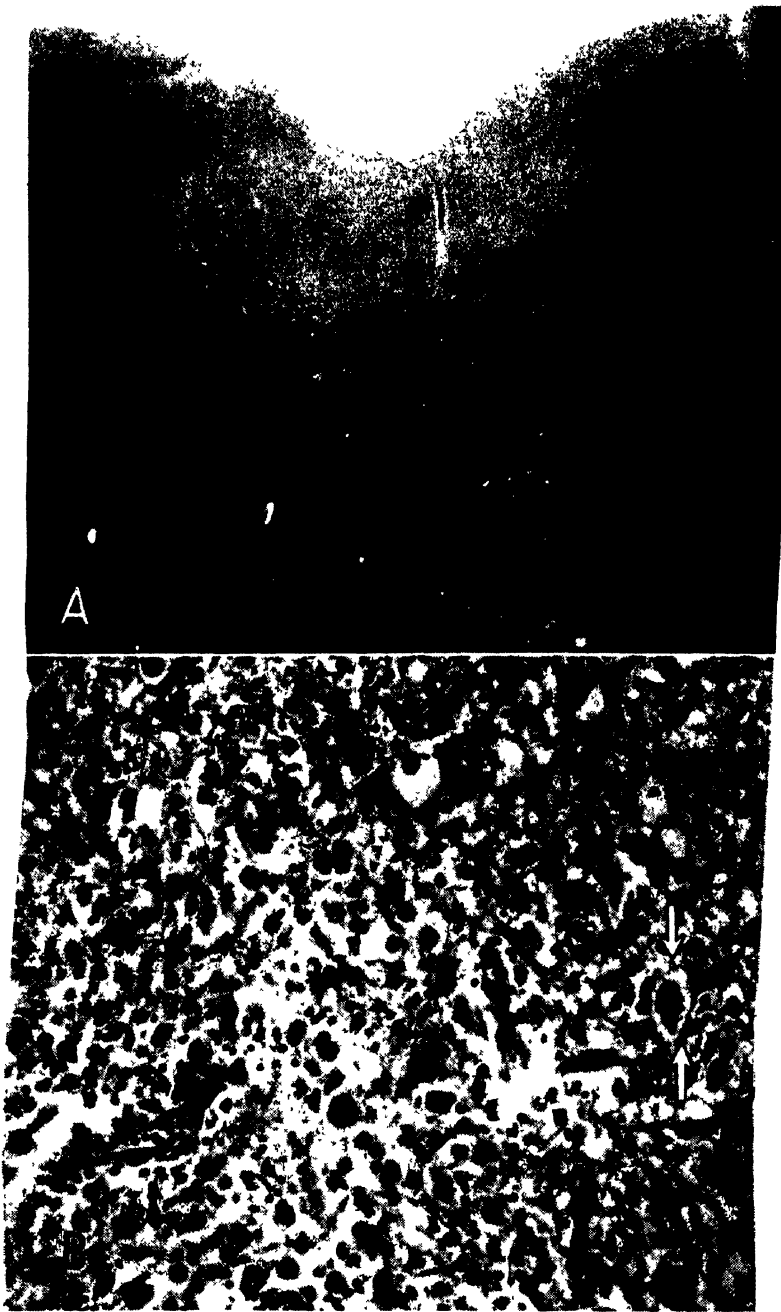


FIG. 4.—*A*, cerebral cortex, illustrating the loss of radial and tangential fibers in a cortical area of inflammation. Weil stain; $\times 18.4$. *B*, tissue showing experimental canine distemper (from a preparation supplied by Dr. De Monbreun). The tissue is from the region of the dentate nucleus. The similarities to the picture described for the dog reported here are discussed in the text. The arrow points to a neuron with a prominent intranuclear inclusion. Hematoxylin-eosin stain; $\times 165$.

vening myelin may at times appear lighter and less dense than in perfectly normal areas. (In the illustration, the partial involvement of the roof nuclei, naturally lighter than the white matter, should not cause confusion.) These "punched-out" areas usually appear, as in the spinal cord, at the margin or near much more severe damage.

The axis-cylinders may show moderate diminution in number and are nowhere as well preserved as they may be in some instances of multiple sclerosis. But the destructive action on the axis-cylinders is not so severe in the cerebellum as in the spinal cord. The usual degenerative alterations may occasionally be found.

There is considerably more gliosis, generally isomorphous in type, in the cerebellar lesions than in the spinal cord. Monster astrocytes are also present. The connective tissue proliferation and the reticulin nets, although present, are much less exuberant than in the spinal cord.

Occasionally in the cerebellar cortex there is an out-dropping of Purkinje cells, without vascular reaction. This change, fairly common in many pathologic conditions, is never severe.

Lesions such as have been described are also present in the white matter elsewhere in the brain—for example, in the optic chiasm and tracts and in the hippocampal commissure. But no damage has been detected in the corpus callosum or the centrum ovale, where Marin-esco, Draganesco and Stroesco⁸ described demyelination.

Scattered in the basal ganglions and cerebral cortex are numerous circumscribed lesions. The usual inflammatory changes are present around the blood vessels, and in the parenchyma there is some glial mobilization, as well as invasion in places by mononuclear cells. The nerve cells are in general excellently preserved, although there may be some increase in satellitosis. Figure 3 *D*, from the ventricular surface of the caudate nucleus, is typical of the lesions found both here and in the cerebral cortex. There is little true destruction of tissue, as found in the spinal cord. Appropriate stains, however, show loss of myelin. Figure 4 *A*, from the neocortex, shows loss of radial and tangential fibers at the site of inflammation. It will be observed that the underlying white matter is entirely normal. It is noteworthy that in these foci in the cerebral gray matter there is intense proliferation of fibrous astrocytes.

Careful search was made for intranuclear inclusions at all levels, but none was found.

COMMENT

This case is pathologically similar to those described by Perdrau and Pugh¹ and by Marinesco, Draganesco and Stroesco²; in all the condition may properly be called "disseminated encephalomyelitis with demyelination." The relation to distemper is not clear. Perdrau and Pugh showed that this form of encephalomyelitis may occur long after, or even in the complete absence of, clinically recognizable distemper.

On the other hand, De Monbreun, studying distemper experimentally, produced and described lesions in dogs which appear to be similar to the demyelination described by others. Dr. De Monbreun sent me a preparation, a field from which is reproduced, with his permission, in figure 4 *B*. The field is from the region of the dentate nucleus of the cerebellum. The extensive damage of tissue, with numerous gutter cells, the inflammatory reaction around the blood vessels and the invasion of the parenchyma by inflammatory cells are clearly in evidence. (The neuron designated by the arrows shows a brilliantly acidophilic intranuclear inclusion, which cannot be well appreciated however in the photograph.) Such a lesion stained for myelin would show demyelination.

It is well established that in distemper as well as in other diseases the inflammatory changes in the nervous system are not of themselves sufficient to induce loss of myelin. The demyelination, according to Perdrau and Pugh,¹ "is not the result of the action of the specific virus of distemper on the central nervous system," but "the virus of distemper plays in this disease of the dog a similar role to that which an acute infection of varying etiology plays in the causation of certain demyelinating diseases of man."

From the work of previously cited authors the conclusion might be drawn that demyelinating lesions may appear in the course of distemper, but also as an indirect, temporally removed consequence, as well as independently of this disease.

Certain features of the case reported here deserve comment. Dif-

ferent types of myelin loss may be seen. First, there is that which is attendant on practically complete destruction of tissue with inflammatory reaction (figs. 1 *B* and 2 *A*). Second, there is the strictly perivascular damage of myelin, appearing around heavily infiltrated blood vessels, where the distended perivascular sheaths have encroached on the parenchyma (figure 3 *A*). Third, there is the more diffuse and often incomplete loss of myelin, sometimes with *état criblé*. In the latter type there may be no inflammatory change in the parenchyma or varying degrees of change up to a very moderate intensity.

In considering the problem of demyelination it is necessary to distinguish sharply between destruction of tissue as a whole and more selective destruction of myelin. Various forms of encephalitis caused by viruses may produce a generally destructive process, in which all tissue elements are severely damaged. There is nothing selective about the process, and the myelin disappears only as a part of the general tissue disintegration. In animals this has been described especially in equine encephalomyelitis.¹¹ There are many instances of myelitis and encephalomyelitis in man in which the observations are similar.

Opposed to the general damage of tissue, in which myelin suffers along with other tissue elements, there may occur in some diseases a selective destruction of myelin. In some instances of multiple sclerosis in which there is a sharp border of demyelination, the axiscylinders within the lesion are essentially intact. Although gitter cells may be present in large numbers, there are generally no "inflammatory" cells. Such a picture, common in multiple sclerosis, was present to some extent in certain areas of the dog described here, especially in the cerebellar lesions. This selective attack on myelin is quite different from the general destruction of tissue found in many diseases proved to have viruses as their etiologic factors as well as in many of unknown cause.

A still different type of demyelination in the dog reported here is the *état criblé*, visible in figures 1 *A* and 2 *B*. This mode of reaction is quite different from multiple or diffuse sclerosis but is comparable to

11. King, L. S.: *J. Exper. Med.* 68:677, 1938.

what is found in combined system disease. The problem of a dietary deficiency superimposed on the original disease process in this dog must remain open.

There are certain similarities to many of the reported cases of neuromyelitis optica, namely, marked involvement of the optic tract and of the spinal cord, with scattered lesions elsewhere. Very intense inflammation with widespread destruction of tissue is a feature of many instances of Devic's disease. It is only the latter cases that resemble the one reported here.

There has been much discussion in the literature regarding the possible nosologic unity of the different demyelinating diseases. To me it seems clear that the disease described here, together with most of the disseminated encephalomyelitides with demyelination recorded in the literature, belongs in a totally different category from multiple sclerosis. Neuromyelitis optica belongs in or very near the former group. Diffuse sclerosis is not a disease entity, but certain of its subdivisions may be related to the first group, others to the second; still others may stand *sui generis*. These views are a matter of opinion only, as are other views expressed in the literature, and must remain so until further etiologic data are available.

SUMMARY

Disseminated encephalomyelitis with demyelination in a dog, of about three months' duration, is presented. There were intense inflammatory changes, with very severe involvement of the spinal cord, and scattered lesions elsewhere, including the optic chiasm and tracts. Different types of demyelination and the accompanying pathologic features are described.

THE CULTURAL AND CLINICAL SIGNIFICANCE IN BOVINE MASTITIS OF NONHEMOLYTIC STREPTOCOCCI THAT FERMENT AESCULIN

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The purpose of this paper is to describe the cultural and clinical significance of group III streptococci and other organisms that ferment aesculin in a small herd from which all cows infected with group I mastitis streptococci (*Streptococcus agalactiae*) were eliminated.

In the simplified cultural classification of the more common varieties of streptococci that are capable of causing mastitis, Minett¹ included in group III those that ferment salicin, mannite, aesculin and inulin, reduce methylene blue milk, and hydrolyze sodium hippurate. He reported that these strains may be responsible for either acute or transient infection with or without a marked change in the character of the milk, and that they have frequently been found in the secretion from apparently normal udders. Diernhofer,² in 1932, described these streptococci and applied to them the name *S. uberis*.

It is apparent from the studies of Plastringe *et al.*³ that in their experience the mastitis produced by aesculin-fermenting streptococci was usually mild and of short duration. Ferguson⁴ described the cultural characteristics of 25 strains of streptococci that split aesculin and stated that on the whole the infections were sporadic and not widespread. Hereafter in this paper, typical streptococci which split aesculin and inulin (not raffinose) and reduce methylene blue milk (1:5,000) will be referred to as group III streptococci.

History of the Herd

The herd concerned in the present study is privately owned, and consists of approximately 19 cows maintained for the production of

milk for family consumption. The cows are kept in an exceptionally modern dairy barn and the stable routine is carefully supervised.

The first bacteriological examination of the fore milk, in 1936, showed that seven purebred Guernsey and Holstein cows were infected with hemolytic streptococci belonging to group I, while two cows were infected in a single quarter with the group III streptococcus. The former animals were immediately disposed of, while the latter were maintained with the normal cows and milked last. Since the elimination of the seven diseased cows, the herd has remained free from infections attributed to group I streptococci.

During a period of 31 months since the first examination of the milk, 15 first-calf heifers have been introduced into the herd; two of these have been disposed of, one on account of sterility, the other because of a lactating rudimentary teat. Four older cows have been eliminated because of low milk production or breeding trouble.

Methods

The fore milk from each quarter of every cow was examined bacteriologically once a month, and the laboratory procedures already reported⁵ were carefully followed. After the samples had been collected at the farm, they were kept chilled and immediately plated in blood agar. The leukocyte films also were prepared at the farm, and the chlorine dilutions set up.

In determining the reaction of the culture in aesculin, the broth was prepared according to the method described by Diernhofer.² The cultures were grown in the broth for 24 to 48 hours and then a drop of a 1 per cent solution of ferric citrate was added. In a positive reaction the solution turned dark brown. In discussing this reaction, Edwards⁶ says:

Harrison and Vanderleck (1909) were able to recognize colonies of *Bacterium coli* in aesculin-iron citrate agar by their black colour. This was due to the fact that the colon bacillus acted on aesculin with the production of glucose and aesculetin, which formed a black compound in the presence of iron.

As noted by Plastringe *et al.*,³ another characteristic of a positive reaction before addition of ferric citrate is the loss of the blue fluorescence present in negative reactions and uninoculated tubes.

In preparing the medium for the cultural classification, the usual

methods were used. As an additional precaution, however, fermentation tests were checked in media to which carbohydrates were added aseptically without subsequent heating of the medium.

RESULTS

Morphology: The streptococci were Gram positive and formed chains of short to medium length. Generally, the cocci were small, round and delicate in appearance.

Growth in Blood-Agar Plate: Following incubation periods of 16 to 48 hours, the colonies appeared to be nonhemolytic. The surface colonies were generally either flat or slightly convex, moist and glistening; yet occasionally the surface growth was slightly opaque. The deep colonies usually were biconvex and produced varying amounts of green pigmentation in the surrounding blood agar. In certain strains the color was very marked and could be seen with the naked eye, whereas in others the pigmentation could be detected more easily under the lens. On prolonged incubation or at room temperatures the green color tended to fade.

Growth in Bouillon: In the original transfer from blood agar, either dextrose or sterile horse serum was added to the broth in order to enhance the growth. The streptococci grew well in the bottom of the tubes as a flocculent deposit with a slightly turbid supernatant fluid.

Fermentation Reactions: The six strains produced about the same reaction in dextrose, the pH varying between 4.7 and 4.9. All of the cultures failed to ferment raffinose; but acid was formed in lactose, saccharose, maltose, mannite, inulin, salicin, sorbitol, and trehalose.

The characteristic reaction in aesculin was demonstrated after incubation for 24 to 48 hours.

Reaction in Bile: Two strains grew sparingly on the surface of 40 per cent bile, and all the cultures were insoluble in bile.

Methylene Blue Milk: The reduction in color with the six strains occurred with a 48-hour incubation period.

Sodium Hippurate: All cultures hydrolyzed sodium hippurate with very little variation in the final reactions.

Other Organisms That React in Aesculin

When the milk is examined bacteriologically in the epidemiological study of mastitis, one may encounter streptococci and micrococci,

other than the group III types, which split aesculin. Moreover, these atypical forms are likewise responsible for either mild or rather severe forms of udder disease. Strains recovered from the right fore quarter of cow 7 and the left fore quarter of cow 8 (see protocols) illustrate this point.

A summary of the more important cultural characters of the group III strains and miscellaneous organisms that ferment aesculin is given in table I.

It will be noted in table I that the six cultures regarded as typical group III streptococci present similar cultural characters, while the strain from cow 7 fails to ferment lactose, saccharose, raffinose, and inulin, or to hydrolyze sodium hippurate. The culture from cow 8 fails to ferment inulin and sorbitol, but grows well in the presence of 40 per cent bile.

Notes on Individual Cows

Cow 1, a nine-year-old purebred Guernsey, calved the last time on May 23, 1937, and was sold in July, 1938, on account of sterility. Group III streptococci were detected in the right hind quarter seven months after the first examination of the fore milk, and thereafter were present in the secretion up to the time of disposal. The bacteria counts varied between 2,000 and 30,000 streptococci per cc. The infection resulted in a distinct induration of the quarter.

Cow 2, a nine-year-old purebred Holstein, calved for the last time July 2, 1938. Group III streptococci were recognized in the milk from the left fore quarter on the first examination (1936) and thereafter on the next nine tests. After the next parturition of June 29, 1937, they were not found in the milk from this quarter on 15 monthly examinations. The character of the secretion was always normal, yet the infection resulted in a slight induration of the quarter. In a monthly examination on December 14, 1938, infection was again apparent in this quarter, as shown by the number of leukocytes (522,280 per cc.), the increase in alkalinity, and the presence of streptococci. Either the original strain was dormant in the udder during a period of 21 months, or the quarter became reinfected with the same cultural type of streptococcus.

Cow 3, an eight-year-old purebred Guernsey, calved last on Decem-

ber 7, 1937. Group III streptococci were cultured in the milk from the right fore quarter on the first examination (1936) and thereafter for 14 months. Streptococci were not detected in the milk on the last eleven monthly examinations. The quarter was slightly indurated, but the secretion showed a normal pH, chloride content, and cell count.

TABLE I

The Cultural Characters of Group III Streptococci and Atypical Organisms Which React in Aesculin

Culture	Morphology	Dextrose	Lactose	Saccharose	Maltose	Mannite	Raffinose	Inulin	Salicin	Sorbitol	Trehalose	40 Per Cent Bile Agar	Aesculin	Methylene Blue Milk (1:5,000)	Sodium Hippurate
Group III streptococci. Strains from cows 1 to 6, inclusive	Small, round, short, chained streptococci, sometimes occurring in small, loose, irregular clumps	4.7 to 4.9	+	+	+	+	-	+	+	+	+	-*	+	+	+
Atypical strain from cow 7	Elongated cocci, usually in pairs, but often appearing in short chains	4.1	-	-	+	+	-	-	+	+	+	-	+	+	-
Atypical strain from cow 8†	Lance-shaped cocci, usually in pairs but often appearing in short chains	4.3	+	+	+	+	+	-	+	-	+	+	+	+	+

* Two cultures show a very slight growth.

† Strain from cow 8 possesses some of the characteristics of *Streptococcus lactis*.

Cow 4, a five-year-old grade Holstein, last calved September 11, 1938. A mild transitory infection of one-month duration was observed in the right fore quarter on the ninth monthly test. Fourteen examinations thereafter failed to reveal the presence of group III streptococci in the secretion.

Cow 5, a grade Holstein first-calf heifer, calved for the second time October 20, 1937. She was sold March 11, 1938, on account of low production and the presence of a rudimentary teat just above the left hind one. This made clean milking difficult, because of occasional

seepage of milk. Group III streptococci were recovered from the milk of the left hind quarter on the first bacteriological examination, conducted four days after parturition. Subsequent monthly tests over a period of seven months revealed their presence in the milk on two occasions. Seven months after parturition, these streptococci appeared in the secretion of the left fore quarter and, up to the time she was dried off, they were observed on six monthly examinations. After the second calving, they were present in the left fore quarter on six out of seven examinations, and in the left hind quarter on two out of five. Induration developed in all the quarters but was more marked in the infected two.

Cow 6, a grade Holstein, calved for the first time on September 29, 1936. Monthly bacteriological examinations of the fore milk throughout two lactation periods at no time revealed the presence of streptococci in the secretion from any quarter. She was dried off August 27, 1938, without subsequent milking, by simply reducing the daily amount of food given. The cow calved for the third time November 7, 1938, with mastitis in the left hind quarter. The secretion was scanty, thick and off color, and the quarter was markedly atrophied. The laboratory examination of the milk on November 21 showed a high alkalinity, a high percentage of chlorine, and a leukocyte count above 13,000,000 cells per cc. The bacteria count was 6,720,000 per cc. and group III streptococci were present in pure culture.

The last negative laboratory examination prior to the calving of November 7 was that of August 8, so that it is possible that the infection occurred following this examination or during the dry period. It should be mentioned here that the sphincters of the teats were exceptionally patent, with seepage of milk after the udder had been cleansed before milking.

Cow 7, a grade Guernsey, calved the first time late in the fall of 1935. The milk was examined twice during the termination of this lactation, and monthly during the succeeding two. Following the last parturition on August 9, 1938, small numbers of aesculin-splitting streptococci were identified for the first time in the milk from the right fore quarter, and on the next monthly examination their numbers had increased. The infection, although mild, had apparently developed during the dry period.

Cow 8, a grade Holstein first-calf heifer, calved for the second time on January 20, 1938. Aesculin-positive, micrococci-like organisms were observed in the secretion from the left fore quarter on the first monthly test. At this time the leukocyte count was 4,066,260 cells per cc. On twelve subsequent monthly examinations, these organisms were identified on eight different tests; whereas on four examinations, if present in the milk, they were in such small numbers that culturally it was impossible to detect them in fore milk samples. The monthly leukocyte count on four examinations was above 1,000,000 cells per cc. Following the second parturition, these organisms have not been detected in the fore milk, middle milk, or strippings on nine monthly tests. Moreover, the other determinations, such as pH, percentage of chlorine, and leukocyte count, indicated normality. The infection, however, resulted in a slight atrophy and induration of the quarters without interfering, however, with the milk production.

DISCUSSION

Of the streptococci that possess the ability to ferment aesculin and inulin, the six strains studied are culturally similar to mastitis streptococci of group III, described by Minett.¹ In the bacteriological examination of samples of fore milk, however, aesculin-splitting streptococci are occasionally encountered that fail to show these group characters. Certain strains differ only in their inability to ferment inulin,⁷ while others show a marked variation in their reactions in the common differential tests. The strain isolated from the right fore quarter of cow 7 offers an illustration of this difference.

Thus it is readily seen that the aesculin medium is useful in the identification of group III streptococci or of closely related types. Moreover, since mastitis streptococci belonging to groups I and II (Minett¹) fail to attack aesculin, its use as a routine diagnostic method is furthermore valuable in the rapid differentiation of the mastitis streptococci.⁶

The results of this study indicate that usually group III streptococci are responsible for a benign infection in the udder with only a slight alteration of the secretion, but that occasionally they may produce acute cases of mastitis (cow 6). Although the majority of the infec-

tions in this herd were mild, with apparent recovery in nearly half of the animals, atrophy and fibrosis of the udder did develop in certain quarters. In this herd infections due either to group III streptococci or to atypical types which react in aesculin were of considerable significance, since over a period of 31 months, eight of the cows were at some time infected, usually in a single quarter. Sporadic cases did occur, since four became infected, and a single quarter of two first-calf heifers was involved at parturition. Yet in most dairy herds the mild infections would have been entirely overlooked unless the fore milk had been tested monthly by as searching a bacteriological examination as was carried out in this herd.

It might appear that cows 2 and 3, which were originally left in the herd, were responsible for the gradual spread of the group III streptococcal infections. It so happens, however, that both of these animals recovered; and cow 2, a high producer (infection reappeared after an interval of 21 months), was kept in a box stall away from the other cows and milked separately. Furthermore, the two first-calf heifers had never been in close contact with the milking cows. In this herd, before milking, the udder of each cow was washed and dried with two separate cloths provided for each animal. These cloths were laundered daily. The attendants washed their hands in warm, soapy water before milking a cow. With all these precautions, it would seem that the chance of direct transmission at milking was very slight.

It is significant that during a period of 31 months after the first bacteriological examination of the fore milk, no new cases of group I infection have appeared in the herd, since usually this is the more common form of mastitis. Minett *et al.*⁸ have suggested that chronic mastitis due to group I streptococci is a contagious disease, and that such streptococci are obligatory parasites usually persisting only in the udder and its secretion. Their observations and those of Plastringe *et al.*,⁹ together with the information obtained in this herd, substantiate the suggestion first proposed by Minett and his coworkers that when cows infected with group I streptococci are removed from a herd, a reservoir for the organisms is eliminated, since the remaining cows can then be maintained free of infection. On the other hand, it may be impossible to develop a streptococcus-free herd until more is

known concerning the source of group III streptococci and the varieties usually responsible for the more acute forms of mastitis.

SUMMARY

The results are given of observations on the cultural and clinical significance of group III streptococci in a small, self-contained herd of cows from which the animals infected with group I streptococci had been eliminated.

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EPIDEMIOLOGY OF LYMPHOCYTIC CHORIOMENINGITIS IN A MOUSE STOCK OBSERVED FOR FOUR YEARS

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Choriomeningitis in a mouse colony is a favorable material for the study of the problem of the effect on a parasite of long association with a given host. The host animals can be easily maintained in large numbers in a relatively small space, and two or three generations can be secured in a year, so that progress is rapid for this type of work. A colony of infected mice has now been observed for 4 years and it is the purpose of this report to describe the changes that have taken place in the disease and its causative agent since the epidemiology was first studied (1).

Methods and Materials

The infected stock, which has been kept in a large metal breeding cage, consisted on the average of 12 to 15 mature females, 2 to 3 mature males, 10 to 20 mice just weaned, and a varying number (usually 1 to 4) of litters of suckling mice. The breeding mice selected from as many different litters as possible were kept for about 7 months and then replaced by animals that had just become sexually mature. Young mice not needed as replacements were discarded at the age of about 4 weeks. The mice removed from the colony were tested for circulating virus from time to time in order to be certain that the disease was still present. Cannibalism occasionally caused losses among the suckling mice. Fighting between the males has been rare. When it occurred the most aggressive animal was removed and replaced by an immature one.

The fertility in the infected stock was considerably below that in the virus-free colony derived from the original infected stock late in 1934 (1). This fact, however, can hardly be attributed to the disease alone, since it has been found that mice breed less regularly when a large number are kept in one cage. In the virus-free colony 1 male and 4 females are kept together in a smaller cage, and under such conditions the mice breed very regularly.

The virus-free mouse stock just mentioned was built up on 6 uninfected mice

(3 males and 3 females) from the mouse colony in which choriomeningitis was discovered in 1934. The progeny of these 3 pairs of mice were carefully tested for the absence of the infection and then cross-bred. This new stock of mice has since remained free from the disease. The mice are uniformly susceptible to choriomeningitis and there is no evidence that their susceptibility has changed since 1935, when the first epidemiological experiments were performed. The health and general condition of the colony are very good.

The infected and the virus-free mouse stocks have been quarantined as strictly as possible. No new mice were added to the colonies, and the diet as well as the environmental conditions was not changed until May, 1938, when the infected stock was taken to Germany.

Further technical details will be given in the text.

A Change in the Mode of Transmission of the Disease

Two modes of transmission of the virus from mouse to mouse were observed in 1935, intrauterine and contact infection. Pregnant females that continued to carry virus in the blood after clinical recovery often transmitted the virus to their embryos. Other mice which did not become infected *in utero*, because their mothers had got rid of the infection, contracted the disease by contact soon after they were born.

Choriomeningitis was essentially a disease of young mice of which many became severely ill and some died during the first month of life. Other mice contracted a subclinical infection. In the period between October 7 and December 11, 1935, for instance, the rate of infection was practically 100 per cent, the morbidity amounted to about 20 per cent, and the mortality to 4.4 per cent in terms of the total mouse population, which then numbered some 150 animals. If the rates of morbidity and mortality had been expressed in terms of the number of immature mice present in the colony, they would have been considerably higher.

The mice infected *in utero* were the only ones to show definite symptoms, while the animals infected by contact shortly after birth merely showed a slightly decreased growth rate. They looked like normal mice to any one not familiar with the exceptionally rapid growth and the large size of the strain of mice used. These observations are based upon the results of a large number of careful tests.

When the epidemiological studies were resumed in 1937, intra-uterine infection had become the only mode of transmission of the virus.

This was probably due to the fact that all of the stock mice, young and old, were carriers of virus. A life-long infection was demonstrated in a number of cases, while the other animals were discarded before the duration of their infection could be determined.

A Decrease in the Severity of the Disease and Its Possible Causes

The most striking change that occurred gradually between 1935 and 1937 was a marked decrease in the severity of the disease. Since it is now subclinical, infected young mice can no longer be distinguished with certainty from uninfected ones in spite of the fact that their tissues contain about the same amounts of virus as before the change occurred.

In view of the fact that certain other virus diseases sometimes change in a similar manner, an attempt was made to determine the cause for the present mildness of the disease. Such an attempt was possible because the mice from the virus-free colony, which had been used for intrauterine infection with stock virus in 1935, were available for comparative experiments on intrauterine infection with virus freshly isolated from the infected stock. On the basis of numerous other observations it could be assumed that the susceptibility of these mice had not appreciably changed since 1935, when intrauterine infection produced a severe disease in the majority of them.

The brains, thoracic and abdominal organs of suckling mice from the infected stock were used as a source of virus. The females needed for the experiments were selected from 5 different litters of mice obtained from the virus-free colony and injected intranasally with stock virus at the age of 1 day. Mice infected in this manner carry virus in the blood for a very long time after clinical recovery (2). 9 females whose blood had been previously tested for virus were bred to 9 virus-free males. 2 young from each of the 9 litters obtained were sacrificed immediately after birth for a test for intrauterine infection. Their brains were removed aseptically and each suspended in 2 cc. saline. The brain suspensions were inoculated intracerebrally into virus-free mice in amounts of 0.05 and 0.05×10^{-3} cc. If these mice, particularly those receiving the higher dilution, developed the disease, it was assumed that the respective litters had become infected *in utero*. This assumption is justified by previous tests which showed that the virus is transmitted either to all of the embryos or not at all. The litters were watched daily for 2 months.

5 of the 9 litters became infected *in utero*, while the other 4 litters did not in spite of the fact that the mothers still carried the virus in the blood 2 weeks after

parturition. This fact suggests that the virus content of the blood may not have been alone responsible for the infection of the embryos of the other mice. The absence of detectable amounts of virus from the blood of another naturally infected mouse that transmitted the virus to its embryos (1, Table III) points in the same direction. It is possible therefore that the transmission of the virus takes place either in the ovaries, which contained relatively large amounts of virus in 2 carriers tested (2), or in the uterus, which likewise may be rich in virus (2), by "growth" through the placenta. The term "intrauterine infection" will be used for the sake of brevity, although it is realized that it may not always be accurate.

TABLE I
Intrauterine Infection in Mice from the Virus-Free Stock

Litter No.	Date of intra-nasal injection of mother at age of 1 day	Date of birth of litter	Number of young	Result
	1937	1938		
1	Oct. 20	Jan. 22	6	1 young became sick and recovered; the others showed decreased growth during the first 2 weeks of life
2	" 26	" 23	7	1 young became sick and recovered; another showed a markedly retarded growth for 4 weeks; the remainder developed normally
3	May 5	" 29	6	No symptoms, but slightly decreased rate of growth
4	Oct. 20	Feb. 1	3	1 young died on the 16th day; the others developed normally
5	" 20	" 22	9	2 young died on the 17th and 37th days; another became sick and recovered; the remainder showed no reaction

Table I shows that the majority of the mice from the 5 litters infected *in utero* failed to become sick. A few of the young however did show symptoms of the disease and 3 of them died. On the whole, the reaction of the animals was intermediate between the severe disease observed in 1935 and the extremely mild infection seen more recently.

The interpretation of this result is not easy. If one assumes that the susceptibility of the virus-free mice is still the same as in 1935, it seems that the pathogenicity of the virus for embryonic mouse tissue has markedly decreased since then. On the other hand, since the disease described in Table I was definitely more severe than that now prevailing in the infected stock, it may be that the mice from the latter

stock now show a higher degree of resistance to intrauterine infection than those from the virus-free colony. In 1935 the susceptibility to intrauterine infection of both kinds of mice was about the same.

The decrease in the severity of the disease resulting from intrauterine infection has been associated with an increase of the virulence of the virus for suckling mice infected by contact shortly after birth, as shown by experiments reported before (2) as well as later in this paper, whereas contact infection in mice older than 2 to 3 weeks is still subclinical as it was in 1935. It would have been of interest to study the effect of contact infection with the stock strain not only in young mice from the virus-free colony, but also in virus-free young from the infected stock. This has not been possible, however, since no mice of the latter type were found.

A Change in the Contagiousness of the Experimental Disease

Experiments on contact infection carried out in 1935 (1, and unpublished experiments) showed that the experimental disease was transmitted by mice of different ages, provided the period of exposure was sufficiently long. Since the disease induced by contact infection was subclinical, it had to be demonstrated by testing the blood for virus or by tests of immunity several weeks after exposure. When the experiments were resumed in 1938, different results were obtained in that the infection very rarely passed from mature mice infected experimentally to normal ones. Suckling mice infected by intranasal instillation of stock virus, however, still transmitted the virus to normal mice during the acute and chronic stages of the disease. Mice infected naturally, young ones as well as old carriers of virus, could likewise transmit the infection. To illustrate these observations some recent experiments will be given here in detail.

The mice infected experimentally as well as the uninfected animals exposed to infected ones were obtained from the virus-free breeding colony, while the naturally infected mice came from the infected stock. As in previous experiments, the mature mice used were all females in order to eliminate the possible sexual transmission of the disease, or the infection by biting in males.

The experiments were conducted as follows: Mice infected naturally or experimentally as indicated in the tables were placed in the same cage with some uninfected animals on the 3rd or 4th day after inoculation or removal from the infected stock. Care was taken that the mice which died from the disease were not de-

voured by their cage mates, since we have recently been able to infect 1 of 6 mice by feeding with virulent mouse brain given on bread. Unless otherwise stated, the animals were kept together for 4 to 5 weeks, after which time they were tested for immunity by intracerebral inoculation with highly virulent virus. The animals that failed to show any reaction after the test of immunity were assumed to have become infected by contact, while the mice that showed characteristic symptoms or died were counted as negative. If the injected mice, to which the normal animals were exposed had shown no signs of illness, for instance, after intranasal or subcutaneous injection with virus, they were also tested for immunity at the same time as the exposed mice to make sure that they had become infected. Control mice of about the same age as the tested animals were included in each immunity test. About 90 per cent of these died, while the remainder developed typical, non-fatal choriomeningitis with characteristic tremors and convulsions. There was not a single control mouse that failed to become sick.

Exposure of Normal Mice to Mature Mice Infected Experimentally.—The details of these experiments are recorded in Table II which shows that the 5-week-old mice usually failed to transmit the infection, no matter by which route they were inoculated.

Since mice injected intracerebrally with virus often do not survive for the period of time that would be necessary for the transmission of the disease (1), the 6 animals injected in this manner with small amounts of virus in Experiment 1 were each given 0.25 cc. hyperimmune guinea pig serum intravenously 3 hours before the virus inoculation. While such serum treatment usually does not prevent the disease, it often renders it non-fatal. Virus was demonstrated in the nasal washings but not in the urine of some serum-treated mice that were sick and ultimately recovered. The mice used in Experiment 4 received no immune serum. They were still sick but evidently recovering when placed in contact with a litter of normal mice on the 9th day after inoculation.

The Influence of the Age at the Time of Inoculation on the Ability of Mice to Transmit the Disease.—The fact that the mothers of litters from the virus-free colony which had been inoculated intranasally with virus at the age of 1 to 7 days always became immune suggested that young mice infected experimentally would transmit the disease more readily than mature mice. That this was the case is shown in Table III.

Mice injected intranasally with virus at the age of 1 day continue to transmit the disease as they grow up in spite of the fact that they show symptoms for only 3 to 4 weeks. This is shown by the experiment recorded in Table IV which was made with 2 mice left over from

the 4th experiment of Table III. They were sick for about 3 weeks, recovered, and appeared quite healthy at the age of 58 days when they

TABLE II
Exposure of Normal Mice to 5- to 6-Week-Old Mice Infected Experimentally with Stock Virus

Experiment No.	Injected mice			Exposed mice	
	Number of mice	Route of inoculation	Reaction	Age at time of exposure	Number of mice infected as evidenced by acquired immunity
1	10	ip*	4 died; 6 became very sick and recovered	5 wks.	1/8†
	4	sc	None; immunized	5 "	0/4
	6	Immune guinea pig serum iv, virus ic	3 died; 3 became sick and recovered	5 "	0/4
2	6	iv	4 died; 2 became very sick and recovered‡	5 "	0/7
3	3	iv	Exposed on 13th day after inoculation when 2 mice had recovered and the 3rd still appeared sick	5 "	0/7
4	2	ic	Just recovering from typical disease	1 day (4-5 mos., mother)	0/5 0/1
5	4	iv	Very slight symptoms followed by quick recovery	1 day (4-5 mos., mother)	0/10 0/1

* ip = intraperitoneally. sc = subcutaneously. iv = intravenously.
ic = intracerebrally.

† 1 out of 8 mice became infected.

‡ The 2 survivors still discharged virus with the urine and nasal secretions at the end of the period of exposure.

were exposed to a newborn normal litter of mice to which they promptly transmitted the disease. At the age of 108 days the 2 mice were again exposed to normal mice of different ages, but for a shorter

TABLE III

The Influence of Age at the Time of Intranasal Infection on the Transmission of the Disease by Contact

Injected mice			Exposed mice	
Age at time of inoculation	Number of mice	Reaction	Age at time of exposure	Number of mice infected as evidenced by disease (in young mice) and acquired immunity
1 day	5	Slight illness; recovery	1 day	5/5
1 "	10	1 died; 5 became sick and recovered; 4 showed no definite symptoms	1 "	8/8
			Full grown mother	1/1
1 "	8	4 died; 4 showed retarded growth	1 day	10/10
			Full grown mothers	2/2
			5 wks.	8/8
1 "	7*	2 died; 2 became sick and recovered; 3 showed only a retarded growth	5 "	7/7
				(Mother of litter injected intra-nasally also became immune)
2-3 wks.	4	2 became sick and recovered; 2 showed no symptoms	2-3 "	0/6
2-3, "	3	1 became very sick and recovered; 2 showed no definite symptoms	5 "	1/5
5 "	5	None; immunized	1 day	0/7
			Full grown mother	0/1
			5 wks.	0/8
5 "	6	1 became sick and recovered; 5 showed no symptoms; all became immune	1 day	0/8
			Full grown mothers	0/2
5 "	6	" "	5 wks.	0/8

* 2 females of this group were used in the experiment recorded in Table IV.

period of time. The disease passed to the majority of the young mice but not to the older ones. This result suggests that young mice contract the infection more readily than mature ones and confirms a previous observation (1).

Infection of Normal Mice by Exposure to Virus Carriers.—That full grown mice from the infected stock, which continue to carry virus in the blood and discharge it with the urine and nasal secretions, can

TABLE IV

Continued Transmission of the Infection after Recovery by Two Mice Injected Intranasally with Virus at the Age of 1 Day*

Age of 2 infected mice when placed in contact with normal mice	Exposed mice		
	Age at time of exposure	Period of exposure and method of testing for infection	Number of mice that became infected by contact
58 days	1 day	Tested for virus in blood on 19th day of exposure	5/5 (All young sick when tested)
	Full grown mother of this litter	Tested for immunity on 25th day of exposure	1/1 (Showed no symptoms)
108 "	1 day	Exposed for 13 days; tested for immunity 2 wks. later	6/8 (The infected mice had shown symptoms)
	5 wks.	" "	0/8

* See footnote to Table III.

transmit the disease to healthy mice has already been reported. This still is the rule, as Table V shows. The disease readily passes from carriers to normal mice of different ages. The majority of the carriers used in these experiments came from litters infected *in utero* that were used in previous experiments (2, Text-fig. 1). They looked quite healthy and could not be distinguished from normal animals.

Comparative Experiments on Contact Infection with Naturally Infected Carriers and Mature Mice Infected Experimentally.—In the following experiments an attempt was made to determine why the disease is often transmitted by healthy appearing carriers but rarely by mature mice infected experimentally. It was not unlikely that the virus content of the nasal secretions, which appears to be more important

for the transmission of the disease than the urine (1), had some connection with this discrepancy. To test this possibility the virus content of the nasal washings from mice infected either naturally or experimentally was determined before and after exposure to normal animals. Each infected female mouse was placed in the same cage with 5 virus-free 5-week-old females, which were tested for acquired immunity after an exposure for 32 days.

TABLE V
Infection of Mice by Exposure to Old Carriers

Old female carriers infected <i>in utero</i> to which normal mice were exposed	Age of carriers at time of exposure	Exposed mice			
		Number	Age when exposed	Number of mice that	
				Showed symptoms	Became immune
	<i>mos.</i>				
2 of Litter C*	10	4†	1 day	2	4
		Mother	4-5 mos.	0	?‡
2 " " D*	10	4†	1 day	2	4
		Mother	4-5 mos.	0	1
3 " " B*	10	1 ♀	4 "	0	1
2 " " "	13	4 ♀	5 wks.	0	4
2 full grown from infected stock	4-5	4 ♀	5 "	0	4

* See Text-fig. 1 in a previous paper (2).

† Virus demonstrated in pooled blood from each group of suckling mice on 19th day of exposure. Mothers not tested for circulating virus.

‡ Death from injury by intracerebral test inoculation.

The nasal washings were taken as already described (2) and tenfold dilutions of them were made in saline. These as well as the undiluted materials were inoculated subcutaneously in amounts of 0.5 cc. into 5-week-old mice from the virus-free stock, one mouse being used for each dilution. It was not practical to titrate the nasal washings by intracerebral inoculation because of the bacteria ordinarily present in them. These were without effect when injected subcutaneously. Since mice inoculated subcutaneously with choriomeningitis virus never show symptoms, the number of infected mice was determined by intracerebral immunity tests made 2 weeks after inoculation.

TABLE VI
The Transmissibility of the Disease by Contact in Relation to Virus Content of Nasal Washings

Experiment No.	Infected mice (♀)				Number of exposed mice (5-week-old ♀) infected as evidenced by acquired immunity
	No.	Mode of infection	Titration of nasal washings		
			Before exposure	After exposure	
1	1	Healthy appearing mouse infected <i>in utero</i> and carrying virus in blood for over 1 year	10 ⁻¹ i*	10 ⁰ i	5/5
			10 ⁻² i	10 ⁻¹ i	
			10 ⁻³ i	10 ⁻² i	
			10 ⁻⁴ ni*	10 ⁻³ i	
			10 ⁻⁵ ni	10 ⁻⁴ i	
	2	" "	10 ⁻¹ i	10 ⁰ i	3/4†
			10 ⁻² i	10 ⁻¹ ?†	
			10 ⁻³ i	10 ⁻² i	
			10 ⁻⁴ ni	10 ⁻³ i	
			10 ⁻⁵ ni	10 ⁻⁴ i	
	3	Mouse injected iv with stock strain 9 days previously. Still sick but recovering when exposed on 9th day	10 ⁻¹ i	10 ⁰ i	1/5
			10 ⁻² i	10 ⁻¹ ni	
			10 ⁻³ i	10 ⁻² ni	
			10 ⁻⁴ ni	10 ⁻³ ni	
			10 ⁻⁵ ni	10 ⁻⁴ ni	
	4	" "	10 ⁻¹ i	10 ⁰ ni	0/5
			10 ⁻² ni	10 ⁻¹ ni	
			10 ⁻³ i	10 ⁻² ni	
			10 ⁻⁴ ni	10 ⁻³ ni	
			10 ⁻⁵ ni	10 ⁻⁴ ni	
2	5	Healthy appearing carrier from infected stock, 4-5 mos. of age	10 ⁻¹ i	10 ⁻¹ i	4/4†
			10 ⁻² i	10 ⁻² i	
			10 ⁻³ i	10 ⁻³ i	
			10 ⁻⁴ ni	10 ⁻⁴ ni	
			10 ⁻⁵ ni	10 ⁻⁵ i	
	6	" "	10 ⁻¹ i	10 ⁻¹ i	1/5
			10 ⁻² i	10 ⁻² i	
			10 ⁻³ i	10 ⁻³ i	
			10 ⁻⁴ i	10 ⁻⁴ ni	
			10 ⁻⁵ ni	10 ⁻⁵ ni	
	7	Same as Nos. 3 and 4	10 ⁻¹ i	10 ⁻¹ i	0/5
			10 ⁻² ni	10 ⁻² ni	
			10 ⁻³ ni	10 ⁻³ ni	
			10 ⁻⁴ ni	10 ⁻⁴ ni	
			10 ⁻⁵ ni	10 ⁻⁵ ni	
	8	" " " " " "	10 ⁻¹ i	10 ⁻¹ ni	0/5
			10 ⁻² i	10 ⁻² ni	
			10 ⁻³ ni	10 ⁻³ ni	
			10 ⁻⁴ ni	10 ⁻⁴ ?†	
			10 ⁻⁵ ni	10 ⁻⁵ ni	

* i = immunized; ni = not immunized. † Mouse died from injury after immunity test.

The details of the experiment are given in Table VI which shows that naturally infected mice (Nos. 1, 2, 5, and 6) in general discharged large amounts of virus over a longer period of time than the other animals infected experimentally (Nos. 3, 4, 7, and 8). This may be the reason why the former mice transmitted the disease more readily than the latter animals.

The urine of the infected mice, which may also play a minor rôle in the transmission of the disease, was not titrated because it was often impossible to obtain more than a few drops of it, and these would not have been sufficient for exact titrations. In other experiments, however, the virus content of the urine often ran parallel with that of the nasal washings, and the same may have been the case in the present tests.

Influence of the Strain of Virus on the Communicability of the Disease.—Since the change in the communicability of the experimental disease may have been due to a change of the virus, it was decided to test this possibility in the following series of experiments. Unfortunately it was not possible to compare the stock virus of 1935 with that of 1938 under the same experimental conditions, because we have not succeeded as yet in preserving choriomeningitis virus in mouse tissue for several years without resorting to animal passage. The latter may markedly alter some characteristics of the virus. In fact, no more than 8 intracerebral passages in mice were necessary to change the pathogenicity for guinea pigs of passage strain B (3). Its virulence for mice likewise differs from that of the stock virus (2). In the following experiments the communicability of the infection induced in mice by the passage strain will be compared with that of virus freshly isolated from the infected stock.

When strain B was isolated from a naturally infected stock mouse in 1935 it produced a contagious disease in 5- to 6-week-old mice. This statement is made with some reserve, however, because the number of mice tested was rather small. In one experiment made with virus from the 1st intracerebral mouse passage, 4 mice from the virus-free colony were inoculated intraperitoneally with virus and exposed to 8 uninfected mice for 32 days. The injected animals were ill from the 6th to the 10th days after inoculation and then recovered. Of the 8 exposed mice 6 became resistant to intracerebral inoculation with highly virulent virus.

The virus used in the present experiments had undergone from 30 to 42 passages in 5-week-old mice. The experiments recorded in Table VII were made with 5-week-old females. They are comparable to those given in Table II and therefore need no special description.

TABLE VII
*Experiments on Contact Infection with the Mouse Passage Strain in
5-Week-Old Females*

Experiment No.	Injected mice			Exposed mice
	Route of inoculation	Number of mice	Reaction	Number of mice infected as evidenced by acquired immunity
1	ic*	1	Dead on 6th day	0/5
	ip	3	None; immunized	
	ic	1	Dead on 6th day	0/5
	sc	3	None; immunized	
2	ic	1	Dead on 7th day	0/5
	in	3	None; immunized	
	Immune guinea pig serum iv, virus ic	5	2 dead on 7th or 8th day; 3 became sick and recovered	0/4
	ip	4	None; immunized	0/4
3	Immune guinea pig serum iv, virus ic	6	3 dead on 7th day; 3 became sick and recovered	0/4
	ip	5	None; immunized	0/4

* ic = intracerebrally. ip = intraperitoneally. sc = subcutaneously.
in = intranasally. iv = intravenously.

Their results were uniformly negative and show that the infection with the mouse passage strain in 5-week-old mice is even less contagious than that with the stock strain. A comparison of the results obtained with the mouse passage strain in 1935 and 1937-1938 gives the impression that its communicability has changed in the course of the serial passages in mice.

In the experiment presented in Table VIII newborn mice were used. 2 litters of mice were injected intranasally with the stock strain and 2

others with the mouse passage virus. On the 4th day after inoculation each group of young together with their uninjected mothers was exposed for 4 weeks to 2 litters of virus-free mice which were tested for immunity at the end of this period by intracerebral inoculation with virus. It can be seen from Table VIII that the stock strain proved more contagious under such conditions than the mouse passage strain and likewise was more virulent for the mice infected by contact.

TABLE VIII
Comparison of Communicability of Stock and Mouse Passage Virus in Newborn Mice

Strain of virus	Mice injected intranasally with virus			Exposed mice	
	Age at time of inoculation	Number of mice	Reaction	Age at time of exposure	Number of mice infected as evidenced by signs of the disease or acquired immunity
Stock	1 day	18	5 died; 5 became sick and recovered; 4 showed only a retarded growth; the remainder presented no definite signs of illness	1 day	18/18 (9 mice showed symptoms, the others a decreased growth rate)
Mouse passage	1 "	18	11 died; 6 became very sick and recovered; 1 showed no signs of illness	1 "	4/13 (The 4 infected mice showed no signs of disease but were immunized)

Effect of a Change in the Environmental Conditions on the Course of the Epidemic

In May, 1938, a collection of mice from the infected stock together with some mice from the virus-free colony were taken to Germany. The animals were shipped in metal cages with screen covers, and these cages had to be kept close together on the trip. The fact that the mice failed to become infected is additional proof that the disease is not highly contagious.

The environmental conditions of the mice in Germany differ from those in America, especially as regards climate and diet. The diet in

America consisted of water, corn, powdered milk, white bread and biscuits, occasionally with lettuce or green alfalfa; whereas the present daily ration comprises water, corn, a special kind of dog biscuit, and rye bread. Green alfalfa is added during warmer weather. Milk is omitted. Both diets appear to be adequate. The method of keeping and handling the animals has not been changed.

The change in the environmental conditions seems to have had no influence whatever on the course and character of the epidemic. The disease is still subclinical. Intrauterine infection appears to be its only mode of transmission, and all of the mice from the infected stock tested have been carriers of virus.

DISCUSSION

The present mildness of the disease in the infected stock appears to have been brought about by a combination of two factors, namely, a change in the mode of transmission of the infection, and a shift in the severity of the disease with regard to the age of the mice at the time of infection. This shift, in turn, seems to have been caused by a decrease in the pathogenicity of the virus for embryonic mouse tissue, and a concurrent increase in the resistance of the stock mice to intrauterine infection. Shifts in the severity of the infection in relation to the age of the host also occur with other virus diseases. Some epidemics of poliomyelitis, for instance, are associated with unusually severe reactions in adults, while the disease in children is milder in contrast to its usual behavior.

If no other shift occurs in the future, one may expect the natural disease to remain mild as long as intrauterine infection represents its only way of transmission. The picture may change, however, when some litters are born virus-free and become infected by contact. In this case one might again find sick mice, unless the resistance to the virus of suckling stock mice has increased also.

The disease has reached a remarkable equilibrium. It no longer causes visible illness, nor is the virus markedly inhibited by the defensive forces of the body. If the virus were a living organism, one might call its present relationship to the host a "perfect parasitism." Theoretically, the mouse is an ideal reservoir host for the virus.

The cause for the change in the communicability of the experimen-

tal disease has not been determined. Since the contagiousness of the disease seems to depend entirely or in part on the virus content of the nasal secretions, it is not unlikely that in 1935 the virus had a greater affinity for the upper respiratory tract of mature mice from the virus-free colony, which decreased with the progressive adaptation of the virus to embryonic mouse tissue.

Hereditary factors, which may have played a part in the changes of the disease and are considered by some investigators to be of great importance in the epidemiology of infectious diseases, have not been studied, because it was not desirable for other reasons to interfere with the natural course of the epidemic by selective breeding, for instance, by establishing genetically pure mouse stocks. The possibility therefore exists that the genetic character of the mice has changed since 1935. In the course of extensive experimental work with mice from the virus-free colony the impression was gained that this stock has not changed genetically. It is not unlikely, however, that the above mentioned change in the resistance to intrauterine infection of the mice from the infected stock was of a genetic nature.

The immunological factors influencing the disease are fundamentally the same as in 1935. It has been noted, however, that the number of immune mice whose blood and viscera contained no demonstrable virus progressively decreased in the infected stock. Today, the very solid immunity demonstrable in all of the stock mice is invariably associated with infection. Their tissues and blood contain surprisingly large amounts of virus (2). This "infection immunity" is of the utmost importance for the epidemiology of the disease, because it permits the virus to be transmitted *in utero* with great regularity and no doubt is chiefly responsible for the long persistence of the disease in the infected stock. It is due to the extremely poor antibody response to the infection in mice as well as to certain other factors which have recently been studied (2).

SUMMARY AND CONCLUSIONS

A small mouse stock in which lymphocytic choriomeningitis is endemic has been observed over a period of 4 years. The disease has persisted during that time, but it has become so mild that it can no longer be recognized by clinical observation. In spite of this fact, all of the stock mice tested, both young and old, carried considerable

amounts of virus in their organs and blood. The females readily transmit the infection to their offspring. Intrauterine infection has become the only mode of transmission of the disease in contrast to the situation in 1935 when a certain number of mice were born virus-free and became infected by contact shortly after birth.

The present mildness of the disease appears to be due to two factors, namely, the change in its mode of transmission just mentioned, and a shift in the severity of the disease with regard to the age of the host at the time of infection. This shift has occurred gradually since 1935 when the mice infected *in utero* were the only ones to become sick. Since 1937, however, the virus is quite harmless for such animals and produces symptoms only in suckling mice from the virus-free stock exposed to contact infection. Evidence is presented which suggests that the shift in the severity of the disease was caused by a decrease of the pathogenicity of the virus for embryonic mouse tissue and a concurrent increase of the resistance to intrauterine infection of the mice from the infected stock.

Another change noted concerned the communicability of the experimental disease. In contrast to observations made in 1935 the experimental infection of mature mice from the virus-free colony is now very rarely transmitted by contact to healthy mice, young or old. Suckling mice from the same stock infected by intranasal instillation of virus, however, readily transmit the disease and continue to do so as they grow up. The same is true for mice infected naturally. The reason for this discrepancy has not been ascertained, but it has been shown that naturally infected mice capable of transmitting the disease in general discharge large amounts of virus through the nose for a longer period of time than mature mice infected experimentally which fail to transmit their infection. It may likewise be of significance in this connection that the virus can lose its communicability by animal passage.

A marked change (chiefly climatic and dietary) in the environmental conditions of the infected stock failed to influence the course and character of the epidemic.

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SEROLOGICAL STUDIES OF SWINE INFLUENZA VIRUSES

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Most of the work with swine influenza virus has been carried out with strain 15, recovered originally in Iowa in 1930. Prior to 1937 this strain was, from time to time, superficially compared with swine influenza viruses obtained in different epizootic outbreaks, and no evidence to indicate immunological heterogeneity among the various strains was detected. Judgment of the identity of the viruses being compared was usually based upon their ability to produce cross-immunity in swine, though some cross-neutralization tests with sera of recovered swine or ferrets failed to detect strain differences either. Swine influenza viruses compared in this way with strain 15 or with one another and considered on the basis of the results obtained to be immunologically identical were strain 14 (Iowa, 1930), strain 17 (Iowa, 1931), strain 18 (Iowa, 1932), strain 19 (Iowa, 1933), strain 20 (Iowa, 1934), and strain 23 (Ohio, 1935).

During the early years of work with human influenza virus, investigators recovered strains from patients in different epidemics and widely separated localities. These viruses from man were assumed, largely on the basis of cross-immunity tests in ferrets, to be immunologically identical. In 1936, however, Magill and Francis (1), using virus-neutralizing serum prepared in a non-susceptible host (rabbit), obtained evidence that their Puerto Rico and Philadelphia strains differed antigenically. Later Burnet (2), Andrewes (3), and Andrewes, Smith, and Stuart-Harris (4) demonstrated serological differences among other strains of human influenza virus. Recently the question of immunologic variation among the large number of strains of human influenza virus now available for study has been thoroughly investigated by Magill and Francis (5, 6) in this country and by Smith and Andrewes (7) in England. The conclusions reached in both investigations were that there is great immunological diversity among strains of human influenza virus and that the virus is antigenically complex. Smith and Andrewes believed that their experiments indicated the existence of at least 4 major antigenic com-

ponents among the 28 strains of virus they studied. They classified the strains, on the basis of their content of the 4 major antigens, into 3 main categories, namely, highly specific strains, relatively non-specific strains, and intermediate strains. Magill and Francis classified their 24 strains into 6 groups as determined by serological similarities or differences and pointed out that the strains which most closely resembled one another were, in general, those from the same epidemic of influenza. Serologically different strains were, however, also recovered from the same epidemic.

These observations concerning serological diversity among strains of the human influenza virus raised the question of whether or not similar variations existed among strains of the swine influenza virus recovered in different epizootics. The experiments reported in this paper were conducted in an attempt to answer the question.

Materials and Methods

Strains of Virus.—The human influenza viruses employed in the present experiments were strains WS, PR8, and Oakham, recovered respectively from cases of epidemic influenza in 1933, 1934, and 1937.¹ The swine influenza viruses used were strain 15 (Iowa, 1930), strain 20 (Iowa, 1934), strain 23 (Ohio, 1935), strain 24 (Nebraska, 1936), strain 28 (Iowa, 1936), strain BC (New Jersey, 1936), and strain 29 (Iowa, 1937).

All strains of virus studied serologically were well adapted to white mice before use in the present experiments and were of such pathogenicity that the supernatant of a 1 per cent infected lung suspension killed all mice inoculated intranasally in less than 5 days. Virus suspensions both for use in neutralization experiments and for the immunization of rabbits were prepared from glycerolated infected mouse lungs.

Sera.—The swine sera were obtained by tail or heart bleeding 11 to 13 days after infection with swine passage swine or human influenza virus mixed with a small amount of a culture of the bacterium *Hemophilus influenzae suis* (8). The swine furnishing the sera were thus in early convalescence.

The rabbit sera were obtained by marginal ear vein bleeding on the 10th and 13th days after intraperitoneal injection with 7 cc. of a 5 per cent suspension of mouse lung infected with either swine or human influenza virus. The 10th and

¹ I am indebted to Dr. C. H. Andrewes for the WS strain, Dr. Thomas Francis, Jr., for the PR8 strain, and Dr. C. H. Stuart-Harris for the Oakham strain.

13th day bleedings from each rabbit were pooled for use in the neutralization tests. This method of immunization differs somewhat from that employed by Magill and Francis (5) in that they bled their rabbits on the 8th day, and they graded their immunizing dose to correspond roughly with the titer of the virus strain being used.

All sera, both from swine and rabbits, were filtered through Seitz pads prior to storage in the refrigerator until used.

Neutralization Tests.—The neutralization tests were conducted in white mice by the technique regularly used in this laboratory (9).

The supernatant of a 2 per cent suspension of glycerolated infected mouse lung was employed as virus, and this was mixed in equal parts with the undiluted sera to be tested. The mixtures were stored for 2 hours in the refrigerator prior to their administration to white mice. 3 etherized mice were inoculated, in testing each serum-virus mixture, by dipping their noses in the inoculum contained in a slightly tilted small Petri dish. The mice were observed for 10 days; all dying were examined at postmortem; and on the 10th day, surviving mice were autopsied and the degree of pulmonary involvement was noted.

Because of the numbers of tests involved, all virus strains could not be studied at one time. The general plan followed, therefore, was to test all of the swine and rabbit sera against each of the strains of virus in turn. With the exception of strain 20, all of the swine influenza viruses were of roughly the same pathogenicity for mice, and the amount of virus administered in each test amounted to between 10 and 100 minimal fatal doses. Strain 20 possessed a slightly lower pathogenicity for mice, and the dilution used in the neutralization tests corresponded roughly to 10 minimal fatal doses. Two of the human influenza viruses, strains PR8 and WS, were of approximately the same pathogenicity as the majority of the swine strains, while the Oakham strain, at the time it was used, roughly corresponded in titer with strain 20 swine influenza virus. No effort was made to titrate the number of minimal fatal doses of virus more closely than by decimal dilutions. In each individual neutralization experiment 5 groups of control mice receiving virus mixed with normal rabbit or swine serum were included, and all of the mice in these groups succumbed of influenza during the 10 day period of observation.

RESULTS

The results obtained with convalescent swine sera are shown graphically in Chart 1 and those with immune rabbit sera in Chart 2. In

the two vertical columns to the left of each chart are listed the animals supplying the antisera together with the strains of virus against which the antisera were prepared. The strain of virus used in neutralization

Serum		Strain of virus									
Swine No.	Immune to virus	S-15	S-20	S-23	S-24	S-28	S-BC	S-29	H-04	H-PR8	HWS
84	S-15								■	■	■
85	S-15								■	■	■
9	S-20								■	■	■
36	S-20								■	■	■
12	S-23								■	■	■
78	S-23	■		■	⊗	⊗			⊗		■
95	S-24								■		■
42	S-28	■						■	■	■	■
97	S-28								■	■	■
69	S-BC								■	■	■
A89	S-BC								■	■	■
77	S-29								■	■	■
B69	S-29			■					■	■	■
20	H-PR8	■	■	■	■	■	■	■			
23	H-PR8	■	■	■	■	■	■	■			■
6	HWS	■	■	■	■	■	■	■			
99	HWS	■	■	■	■	■	■	■	■	■	

CHART 1. Cross-neutralization tests in mice with convalescent swine serum.

■ All mice in the test died of influenza. No neutralization.

■ Mice survived but showed extensive lung lesions at autopsy on 10th day. Slight neutralization.

■ Mice survived and showed only scant lung lesions at autopsy on 10th day. Partial neutralization.

□ Mice survived and showed no lung lesions at autopsy on 10th day. Complete neutralization.

⊗ Not tested.

tests with the various sera is given at the top of each of the other vertical columns.

As shown in Chart 1 all swine convalescent sera, regardless of the

Serum		Strain of virus											
Rabbit No.	Immune to virus	S-15	S-20	S-23	S-24	S-28	S-BC	S-29	H-04	H-PR8	H-WS		
44	S-15												
45	S-15												
63	S-15												
71	S-BC												
75	S-BC												
76	S-BC												
32	S-23												
74	S-23												
34	S-24												
78	S-24												
79	S-24												
29	S-28												
77	S-28												
50	S-29												
51	S-29												
53	S-29												
31	S-20												
55	S-20												
56	S-20												
24	H-PR8												
25	H-PR8												
46	H-PR8												
66	H-PR8												
68	H-PR8												
22	H-WS												
58	H-WS												
59	H-WS												
60	H-WS												

CHART 2. Cross-neutralization tests in mice with sera of immunized rabbits. Designation of results same as Chart 1.

strain of swine influenza virus from which the animals supplying the sera were convalescent, neutralized all strains of the swine influenza virus. In like manner, the 3 human viruses tested were neutralized by the sera of swine recovered from infection with either the WS or PR8 strains of human influenza virus. Between the human and the swine strains the serological relationship found to exist was variable; only one of the human virus antisera (swine 99) had any appreciable neutralizing effect on any of the swine viruses. In the reverse direction, however, most of the swine virus antisera partially neutralized the WS and Oakham strains. The PR8 strain was neutralized partially by only one of the swine virus antisera. These findings taken alone would indicate that each strain of swine influenza virus was serologically like all of the other swine strains in the present experiments. The 3 human viruses would also have to be considered alike on the basis of the results with the human virus antisera. However, consideration of the neutralization tests with the human viruses and swine virus antisera makes it evident that the Oakham and WS strains behave quite differently from the PR8 strain, and it would seem that these two strains are immunologically more closely related to swine influenza virus than is the PR8 strain. The important feature of the data given in Chart 1, so far as they concern the present experiments, is that no evidence is furnished to indicate serological heterogeneity among the 7 strains of swine influenza virus under study.

The results with virus-neutralizing rabbit sera recorded in Chart 2 are not as clear cut and constant as were those with swine sera. Among the swine influenza viruses, strains 15 and BC produced potent antibodies in rabbits both for themselves and for all heterologous swine strains as well, but were, as a rule, neutralized only partially or not at all by antisera prepared against the heterologous swine viruses. Strain 20, on the other hand, was readily neutralized by sera prepared against all of the other swine strains and the PR8 human strain but itself produced antibodies poorly or not at all for the heterologous swine viruses. Strain 29 resembled strain 20, though here one of the 3 rabbits used (rabbit 53) produced fairly good neutralizing antibodies for heterologous strains. The 3 remaining swine strains resembled strains 15 and BC in that they produced antibodies in

rabbits effective at least partially against all the other swine viruses but differed in that neutralization of the heterologous viruses was seldom complete as with the strain 15 and BC antisera. There are exceptions to this attempted classification, obvious from consideration of Chart 2. This suggests that at least some of the differences noted may be more dependent upon variations among the individual rabbits used than among the strains of swine influenza virus under study.

The rabbit antisera more effectively differentiated between the swine viruses and the WS and Oakham strains of human influenza virus than had the swine antisera. With the exception of PR8 antisera against strain 20, there was little cross-neutralization between swine and human strains. Furthermore, the rabbit antisera rather clearly differentiated between the PR8 and WS strains of human virus, something the swine antisera had failed to do.

DISCUSSION

It is difficult to reconcile the results obtained with swine convalescent sera and those obtained with sera of immunized rabbits as to their relative significance in denoting serological homogeneity or heterogeneity among the strains of swine influenza virus studied. If the results with swine convalescent sera were the only ones available, it would be simple to conclude that the 7 swine viruses were serologically alike and possessed the same general antigenic composition and pattern. If, on the other hand, only the results with sera of immunized rabbits were to be considered, it would be necessary to recognize the existence of antigenic variations among the swine influenza viruses. Thus, from the rabbit serum results, strains 15 and BC, which appear antigenically alike, differ from strains 20 and 29 in that they are not neutralized by antisera prepared against strains 20 and 29. Antisera prepared against 15 and BC do, however, neutralize strains 20 and 29. The remaining 3 strains lie intermediate between these two groups, though resembling strains 15 and BC most closely in their serological behavior. The classification which rabbit antisera seem to have made among the strains of swine influenza virus studied corresponds, in a way, with that into which Smith and Andrewes (7) grouped their human viruses. Strains 20 and 29 could be designated, according to this arrangement, as "specific" strains in

that they produce antibodies that are largely effective against only the homologous strains. Strains 15 and BC would correspond to Smith and Andrewes' "non-specific" or "master" strains, viruses which produce antibodies effective against the whole group of swine influenza viruses. The remaining viruses, strains 23, 24, and 28, would be classified as "intermediate" strains, though resembling the "non-specific" strains more closely than the "specific." There are, however, several individual exceptions to this rather general classification. For instance, rabbit 53, immunized with strain 29, developed antibodies that neutralized heterologous swine strains almost as broadly as sera prepared against 15 or BC. This serum also neutralized the WS strain human influenza virus completely, the only one of the anti-swine virus rabbit sera to be completely effective against any of the human viruses. In like manner, the antisera of rabbits 79 and 55 prepared respectively against strains 24 and 20 were unusual, when compared with antisera of other rabbits immunized with the same viruses, in their capacity to neutralize heterologous strains of swine influenza virus.

It is not believed that the various differences among the swine viruses, detectible by antisera prepared in rabbits, are due to differences in antibody titers of individual rabbit sera used, because frequently the differences are in the wrong direction to be accounted for in this way. Rather it would seem that rabbit antisera actually detect strain differences that are not reflected in convalescent sera of the natural host animal. Such differences are probably of no practical importance so far as the natural disease, swine influenza, is concerned and have an academic interest only in that they indicate a variation in the antibody response to the virus of a susceptible and a non-susceptible host.

Since, in the natural host of swine influenza, all strains of the virus give rise to an antibody response indicative of antigenic homogeneity, the question is raised as to whether the swine serum or the rabbit serum results should be more seriously considered in arriving at a decision as to whether the swine influenza virus strains studied are serologically alike or different. There can be no doubt that in rabbits the various virus strains give rise to antibodies with differing virus affinities. However, in the rabbit, swine influenza virus exhibits no evidence of pathogenicity and is probably not infective in the sense

in which that term is usually applied to indicate invasiveness and persistence of an infective agent in a susceptible host. In all probability, swine influenza virus acts in a manner analogous to that of any other invasively inert, antigenic substance in eliciting a specific response in rabbits. Thus if the swine influenza virus is antigenically complex, as Magill and Francis' (5) and Smith and Andrewes' (7) findings indicate the human influenza virus to be, then one might anticipate that the first antibody response of rabbits would be to the dominant or most readily accessible of the swine influenza virus antigens. In swine, on the other hand, where immunity follows actual multiplication of the virus within the host, invasion of susceptible cells by the virus, and finally, destruction or inactivation of virus at the time of recovery, one might expect an immunological host response to all of the various antigens comprising the virus. It seems entirely possible that the apparent discrepancies between the swine and rabbit serum findings may be accounted for by this difference in the mechanism whereby the virus-neutralizing antibodies are produced in a non-susceptible animal, the rabbit, on the one hand, and in a susceptible host, the swine, on the other. On such a basis, antisera prepared by the infection of swine with virus would be considered to reflect the entire antigenic content or composition of the virus, while antisera prepared by the injection of virus, infectively inert for rabbits, into these animals would be thought of as reflecting the arrangement, within the virus, of the components responsible for mouse pathogenicity. Such an explanation of the findings would orient the apparently discrepant results obtained with swine and rabbit antisera. The conclusion to be reached under this interpretation would be that the various strains of swine influenza virus studied are similar in their antigenic composition but that they vary among themselves either in the arrangement of their common antigenic components or in the situation, within the virus, of the components responsible for their mouse pathogenicity.

CONCLUSIONS

1. Cross-neutralization tests with sera from swine recovered from infection with swine influenza indicated the serological identity of 7 strains of swine influenza virus obtained from different sources.
2. Cross-neutralization tests with sera from rabbits, immunized

to swine influenza virus, exposed serological differences among the same 7 swine influenza virus strains. Two strains appeared to be serologically similar and were characterized by the ability to produce effective homologous virus-neutralizing sera which were, however, poor or ineffective against the heterologous virus strains. Two other strains were also serologically similar but produced antibodies effective not only against themselves, but against all heterologous strains as well. The remaining 3 strains were intermediate in their ability to produce heterologous virus-neutralizing antibodies.

3. The human influenza viruses included, especially strains WS and Oakham, were most effectively differentiated serologically from the swine influenza viruses by rabbit antisera.

4. The suggestion is advanced that swine antisera express the antigenic composition of the swine influenza viruses, while rabbit antisera reflect either their antigenic arrangement or the arrangement of the components responsible for their mouse pathogenicity. On this interpretation the 7 strains of swine influenza virus studied would be considered to have similar antigenic compositions but differing antigenic structures.

5. The serological differences among strains of the swine influenza virus, detectible by rabbit antisera, are probably of no practical significance so far as the natural disease, swine influenza, is concerned.

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A COMPARISON OF THE LUNG LESIONS PRODUCED IN LABORATORY ANIMALS BY *ASCARIS LUMBRICOIDES* OF SWINE

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Davaine (1863) observed the hatching of *Ascaris lumbricoides* eggs in the intestine of rats. While repeating these observations Stewart discovered that *A. lumbricoides* larvae migrate through the liver and lungs of rats (1916) and mice (1916a). Many later reports have expanded our knowledge of ascaris hatching and wandering and have created the impression that this migration, with resulting lung lesions, occurs with equal facility in a number of experimental animals. The purpose of the work to be reported was to compare rats, mice, chickens, cats and other animals with guinea pigs in their susceptibility to lung infection with *A. lumbricoides* larvae following the administration of known infective eggs. A brief abstract of this material has already been presented (Graham, 1934).

Materials and Methods

Ascaris lumbricoides eggs used in these studies were secured from screened and washed pig feces by brine flotation. They were washed in distilled water, placed in 125 cc. Erlenmeyer flasks in 15 cc. of 0.2 per cent formalin (1 cm. deep); the flasks were plugged with cotton and incubated at 29-30°C. or at room temperature until they were demonstrably infective for guinea pigs in doses of 100 eggs.

Recently, Stoll (1933) has emphasized the fact that embryonated ascaris eggs must pass through a post-embryonation lag or maturation period before becoming infective. He demonstrated a correlation between the dosage of infective eggs and the resulting lung lesions in guinea pigs. Only infective eggs in known dosages have been used in the present study. Doses of 600 or less eggs were counted with the aid of a microscope; doses of 1,000 eggs or more were prepared by a careful

dilution technique. Guinea pigs were used as standard test animals since it was with them that Stoll devised his gradation scheme, which I have followed, of classifying as one plus to four plus (+ - - - - + + + +), lung lesions which range from a single petechial spot to intense, confluent, ecchymotic areas which frequently resemble complete hepatization of the lungs. The readings have been made upon freshly killed animals. Following etherization, the abdominal cavity was opened and the vena cava and dorsal aorta cut to permit free bleeding. After washing, the thoracic cavity was opened and the exposed lungs classified with a minimum of handling.

Three strains of rats have been utilized in these studies. One was a yellow hooded strain which has been employed in experiments Ia, Ib, and VIII; another was the albino strain maintained in this institute and used only in experiment II; and the third was an albino strain used in experiments III, IV and V. No differences were attributable to the use of these various rat strains.

RESULTS

I. Ascaris lumbricoides in Rats and Guinea Pigs

Experiment Ia

Four rats, five weeks old, were fed *A. lumbricoides* eggs from a culture (A) which had proved fully infective for guinea pigs; two received 500 eggs each on dry bread and two received 3,000 eggs each in the same manner. At autopsy, six days later, one of the rats fed 500 eggs showed no lung lesions, the other only a few, being classified as + lesions. The two rats fed 3,000 eggs also showed few lesions and were similarly graded. The comparisons of these lung readings with those of the guinea pigs, which showed lung readings of +, +(+) , + + + , and a very heavy + + + + from feedings of 100, 500, 5,000 and 50,000 eggs respectively, indicated a dissimilar susceptibility of these two hosts for *A. lumbricoides*.

Experiment Ib

Eight young rats (av. wt. = 130 gm.) in a new series were each fed 7,500 *A. lumbricoides* eggs on bread after a 24-hour fasting period. Eggs from this culture (B) were also fed to three guinea pigs in doses of 200, 600 and 7,500 eggs respectively. Food was withheld from the rats for about 2½ hours after the feeding. At autopsy on the sixth day after infection, although the guinea pig autopsies revealed lung lesions classified as +, +(+) and a very heavy + + + + , indicating that a highly infective egg culture had been used, seven of the eight rats fed showed only slightly positive lungs and one was negative. The results of this

experiment closely resemble those of experiment Ia. A dose of 100 eggs in a guinea pig produced lesions in the lungs that were the equivalent of those produced in rats by a dose 75 times as large.

Experiment II

In this group of rats (three months old), *A. lumbricoides* eggs were injected into the stomach by means of a hypodermic syringe following laparotomy. Four rats received 120, 500, 5,000 and 10,000 eggs respectively. Similar doses from the same egg culture (C) were fed to four guinea pigs. The lung readings recorded in table 1 were made at autopsy on the sixth day.

Experiment III

Since etherization might have helped to prevent infections in the rats of the preceding experiment, a further test was made to eliminate this possibility as well as to extend the dosage range. Twenty-one rats were used, all but three of which averaged 165 grams in weight; these three averaged 265 grams in weight and

TABLE 1

<i>A. lumbricoides</i> eggs used	Lung lesions at 6th day	
	Rats (injected into stomach)	Guinea pigs (fed)
120	(+) single spot	+
500	—	++
5,000	+	++++
10,000	+	++++

were included in the group receiving eggs by stomach injection. They were fasted for 20 hours prior to injection with 10,000, 20,000 and 40,000 eggs respectively. Three similarly fasted younger rats were also injected with these dosages, as were three non-fasted animals.

The twelve rats which were fed the eggs were fasted 24 hours. In half the feedings, the *A. lumbricoides* eggs were placed on dry bread. Pairs of rats were fed 10,000, 20,000 and 40,000 eggs respectively. These feedings were duplicated using cheese instead of bread. The eggs were placed in a depression on a small bit of cheese and the fluid permitted to evaporate before feeding. The eggs were from culture C, used previously in experiment II. One rat of every pair was anesthetized for a period equivalent to that required for making a stomach injection (10–15 minutes) immediately after it had finished eating. As controls, two pairs of guinea pigs were fed 100 and 500 eggs respectively and of each pair one had been fasted and the other given food prior to receiving the eggs.

The lung readings made on the rats at autopsy six days later were all slightly positive, except that one large fasted rat which had received 20,000 eggs by

injection showed no lung lesions. Although the lesions observed were gradable as +, in general the 40,000 series was slightly heavier than the 20,000, and this in turn heavier than the 10,000 group. There was no observable difference between the rats fasted or not fasted in relation to stomach injection of eggs; nor did these differ from those receiving the eggs on food. No distinction could be made between rats etherized after feeding or those not etherized; nor did the duplicate series using cheese to carry the eggs differ from the bread-fed series.

The four guinea pig controls were all positive, even with the 100 egg doses; the two fed 500 eggs were somewhat more heavily infected. No differences were discernible between the fasted and the fed animals.

This experiment makes clear the distinction between the infectibility of rats and guinea pigs with *A. lumbricoides* eggs. Doses of 100 eggs in guinea pigs resulted in lung lesions comparable to those produced by 40,000 eggs in rats.

Fecal collections from the seven rats given 40,000 eggs were made. Dilution counts indicated a recovery of embryonated eggs and larvae ranging from 28 to 40 per cent. Hatched larvae composed 44 to 77 per cent of this total. No significant differences were observed between the rats given eggs by injection and those fed eggs.

Experiment IV

A further test, with a larger maximum dose, was made on three rats weighing 225 grams each, by injection into the stomach of 18,000, 36,000 and 72,000 *A. lumbricoides* eggs respectively. At autopsy six days later, the rat given 72,000 eggs was classified as a +, slightly heavier than the rat given 18,000 eggs. The rat given 36,000 eggs was killed on the third day, being moribund presumably due to an intercurrent infection. It had a few lung lesions. Control guinea pigs fed respectively 87, 500 and 1,000 eggs from the same culture (D) all had positive lungs at autopsy, the severity of lesions corresponding to the dosage of eggs fed.

A dose of 72,000 infective eggs in a rat resulted in lung lesions of a degree comparable to a dose of 87 eggs in a guinea pig.

Experiment V

Fourteen rats, averaging 240 grams in weight, received stomach injections of 11,500 *A. lumbricoides* eggs each. Three guinea pigs were fed 100, 500 and 5,000 eggs respectively as a control on the egg culture (E).

A rat was killed daily for examinations which covered the intestine, liver, lungs, blood (citratd: obtained by cutting the portal vein and vena cava), and Baermann isolations of the lungs and liver. The intestine was examined from only the first two rats; no macroscopic lesions were found in either case. Five, no larvae, and three larvae were recovered from the blood of the rats examined on the first three days.

The lung lesions, all classified as +, were readable from the fourth to the thirteenth day, but were definitely regressive beginning on the tenth day. A bronchopneumonia present in some of the rats did not complicate the lung readings. Baermann isolations of the lungs yielded a single larva on the sixth day. Negative on the seventh day, isolations then yielded 3-4 larvae per rat from the eighth to the eleventh day inclusive. This lag, after readable lesions appeared until isolations yielded larvae, may indicate the migration of more larvae into the lungs or more effective isolation of the enlarged larvae.

The livers of the rats killed on the first and third day showed a slight congestion. Baermann isolations revealed one larva on the first day, two on the fourth, four on the fifth and one on the eighth. After the fourth day the larvae were found to be considerably enlarged.

All lung readings from the first positive day, the fourth, to the eleventh day were made with no difficulty. The positive readings on the twelfth and thirteenth day were made on very faint lesions which were rapidly being resolved. Since the lesions were uniform, it was evident that satisfactory readings may be regularly obtained on the sixth day. The guinea pig controls on the egg culture revealed lesions from a dose of 100 eggs and graded up to a +++ reading from 5,000 eggs. This experiment, like those preceding, showed that while lung lesions occurred in rats following large doses of infective *A. lumbricoides* eggs, the lesions produced were neither numerous nor correlated to any extent with the size of the egg dosage; whereas, in guinea pigs the intensity of the lesions followed closely the number of eggs fed, especially at the lower dosages.

II. Ascaris lumbricoides in Other Animals (*Mice, Rabbits, Chickens, Cats, a Dog and an Opossum*)

Experiment VI

Fifteen young adult female mice were divided into five lots of three each to be given oral injections of 100, 500, 1,000, 5,000 and 10,000 eggs of *A. lumbricoides*. The mice were lightly anesthetized with ether and the injections made with a tuberculin syringe and a blunted, slightly curved 18 gauge needle. Guinea pigs were fed similar doses of eggs from the same culture (B). Results obtained at autopsy on the seventh day are presented in table 2.

The classification of one of the mice given 1,000 eggs, although probably a +, remains doubtful because the pleural cavity was filled with blood. Except for a single mouse of the 10,000 group, presumptively classified as a ++, all had very few lesions in comparison with guinea pigs given similar doses.

From this experiment, it appears that mice are somewhat better than rats for the demonstration of lung lesions due to ascaris migration. Although 100 eggs produced readable lesions, the heavier doses resulted in only light lesions in comparison with the lesions produced in guinea pigs from the same egg dosages.

Experiment VII

The egg culture (B) used in the preceding experiment was employed at the same dosage levels, plus an additional 50,000 egg dose, for feedings to Rhode Island Red chickens (17 days old) and to rabbits. Autopsies were made at seven days and the results recorded in table 3 were obtained.

The chicken, showing a very light + lung after a dose of 50,000 eggs, can only be classified as extremely poor for ascaris migration. Rabbits, on the other hand, are very effective for this purpose. The gradation of lung lesions was apparent in every rabbit used, even in those fed 100, 500 and 1,000 eggs where

TABLE 2

<i>A. lumbricoides</i> eggs used	Lung lesions at 7th day	
	Mice (oral injection)	Guinea pigs (fed)
100	++;	+
500	++;	++
1,000	++; (+)?	+++
5,000	++;	++++
10,000	++; +(+)	++++

TABLE 3

<i>A. lumbricoides</i> eggs used	Lung lesions at 7th day	
	Chickens (fed with pipette)	Rabbits (fed with pipette)
100	—	+
500	—	+
1,000	—	+
5,000	—	++
10,000	+	+++*
50,000	+	++++

* A control was caged with this rabbit and showed + lung lesions at autopsy.

the lung lesions were graded as + lesions. In this respect, rabbits seem to be equal to guinea pigs, for light and heavy doses produced lesions which compared favorably with those in guinea pigs for intensity of lesions. The finely stippled lesions in the rabbit tend to remain as discrete, punctate petechiae showing little or no tendency to fuse or become blotchy as in the case of the guinea pig. This difference favored the latter for ease of reading and classification of the lesions. For reasons of economy and convenience in handling and feeding, guinea pigs are more satisfactory animals to use for an optimum test of infectivity.

A control rabbit, caged with the animal given 10,000 eggs, showed + lung lesions at autopsy comparable to the animal fed 100 eggs. Another control, caged alone, showed a few hemorrhagic spots which were not typical of ascaris and may have been due to handling.

Experiment VIII

A further test of lung infectibility was made using chickens, rats, cats and guinea pigs. The chickens were Rhode Island Reds, one month old; the rats were year old females; and the cats were five and one-half months old. All except the guinea pigs, which were fed with a pipette as usual, were given *A. lumbricoides* eggs (culture G) by means of a #5 soft rubber catheter attached to a 1 cc. tuberculin syringe. The catheter was flushed with water following delivery of the eggs to ensure complete administration of each dose. The chickens, rats and guinea pigs were given doses of 100, 500, 5,000, 25,000 and 50,000 eggs, while the three cats were given only the three larger doses. The animals were examined

TABLE 4

<i>A. lumbricoides</i> eggs used	Lung lesions at the 6th day			
	Chickens (fed by catheter)	Rats (fed by catheter)	Cats (fed by catheter)	Guinea pigs (fed by pipette)
100	-; -; -	-; -; -		+; +; +
500	-; -; -	-; -; (+)		+(+); +(++)
5,000	(+)	(+); (+)	-	++++
25,000	(+)	-; (+)	-	++++
50,000	(+)	-; (+)	-	++++

at autopsy on the sixth day after infection and the lung readings recorded in table 4 were made.

As indicated in table 4, cats failed to show lung lesions, even with a dose of 50,000 infective eggs, whereas guinea pig lungs were severely damaged by an egg dose one-tenth as large. The chickens were negative at the two lower doses, showing only minimal positives (+) at 5,000, 25,000 and 50,000 egg doses. The rats showed few lesions from the heavy doses, all being minimal (+). The reading of one of the rats given 500 eggs as a (+) represented an uncertainty as to whether it was a negative or a positive lung. The lung was badly scarred from a chronic pneumonia.

The results of this experiment resemble those of previous tests of chickens and rats with *A. lumbricoides* in regard to lung infectibility. Rats and chickens are essentially poor hosts when compared with guinea pigs.

Experiment IX

A female dog (three years old) was fed 51,000 eggs from a culture (F) which had yielded about 50 lung lesions from a dose of 72 eggs in a guinea pig as well

as a ++ reading from 500 eggs in another guinea pig. The eggs were fed in two doses in double gelatine capsules. Autopsy on the eighth day after infection revealed no lung lesions. Samples of the liver and lungs, finely chopped and isolated in the Baermann apparatus, proved to be negative for larvae upon examination.

Experiment X

The capture of a female opossum (*Didelphis virginiana*), apparently a young adult, on the grounds of the institute afforded an opportunity to test the lung infectibility of this mammal with *A. lumbricoides*. A dose of 75,000 eggs from an infective culture (H) was administered to the opossum by means of a catheter. Three control guinea pigs were given 100, 600 and 5,000 eggs respectively. At autopsy on the eighth day, the guinea pigs showed lung lesions classified as +, ++ and +++ respectively, while the lungs of the opossum were without a single blemish. The only nematodes found in this animal were eight *Physaloptera turgida* in the stomach and about 150 oxyurids, *Crusia tentaculata*, in the cecum.

DISCUSSION

The foregoing results are enlightening with respect to the production of macroscopic lung lesions in test animals subsequent to the administration of measured doses of infective eggs of *Asearis lumbricoides* of swine. They permit certain conclusions to be drawn which may be accepted as replacement, in part at least, of certain ill-defined conceptions currently held in regard to the ability of ascaris larvae to make the pulmonary migration. For example, Chandler (1930, p. 337) referring to *A. lumbricoides* says: "The migration through the lungs takes place readily in rats, mice, guinea pigs and other rodents," and Ransom and Foster (1920, p. 3) believed that: "Our investigations have shown that the parasites migrate through the lungs in the guinea pig, rabbit, sheep, goat, pig, and presumably man, as well as in the rat and mouse." While it is recognized that "lung migration" may not be strictly interpreted to mean "production of lung lesions" since Fülleborn (1921) has demonstrated that larvae may pass through the capillaries to the left heart and the systemic circulation, a perusal of the literature reveals a usage of these two terms which is practically synonymous. Ransom and Foster (1920, p. 11) indicated that the larvae were halted by the lung capillaries and escaped into the air vesicles following hemorrhage of the arterioles although they recognized that some larvae might be swept through into the arterial system.

In the absence of experimental tests the inference has been drawn that ascaris larvae migrate and produce lung lesions with ease in many common laboratory animals. Although this is true for guinea pigs and rabbits, the present study shows that rats are inferior hosts¹ for the lung migration of *A. lumbricoides* larvae when compared with guinea pigs. Known infective eggs in measured doses produced differing degrees of lung hemorrhage in guinea pigs and rats which were extreme in their contrast and remarkable in their consistency. Similarly, mice were but little better than rats, differing to the extent that they showed lung lesions with small doses but failed at higher doses to approach guinea pigs in this respect. Stewart (1916 and 1916a) induced death in rats and mice by heavy feedings of pig and human ascaris eggs. Likewise Ransom and Foster (1920) observed mice to die from two to nineteen days after feedings of unknown numbers of *Ascaris suum* [= *A. lumbricoides*] eggs on bread. Apparently overwhelming doses of eggs must be used to produce fatal lung damage in rats for 40,000 and 72,000 eggs were ineffective in the present study. The fatal dose for mice must also be large for five and ten thousand *A. lumbricoides* eggs did not prove fatal for mice in seven days in the present case; in fact, these doses only produced lesions the equivalent of those regularly produced by 100 eggs in the guinea pig. Concerning tests of parasite mortality in the mouse, Stewart (1916a) reported that, "An average dose of eggs consisted of about 5,000, the average number of larvae found in the lungs has certainly not exceeded 50—that is, only 1 in 100 survives," thus indicating that their susceptibility is not great.

In the present study chickens have been demonstrated to be highly refractory to ascaris migration as shown by the paucity of lung lesions even when fed 50,000 fully infective eggs. Payne, Ackert and Hartman (1925) fed unknown numbers of *A. lumbricoides* eggs to rats and a young chick and subsequently found larvae in their livers and lungs a few days later. Danheim (1925) also fed embryonated eggs of *A. lumbricoides* (dosage not stated) to a young chicken, in the liver and lungs of which larvae were found three days later.

Rabbits have been found comparable to guinea pigs in respect to lung damage from feedings of known doses of *A. lumbricoides* eggs.

¹Footnote: The term "hosts" is used in a restricted and special sense.

The contamination and subsequent infection of a control rabbit caged with an animal given 10,000 eggs is interesting in the light of Martin's (1926) experience where he observed such heavy contamination from eggs passed in the feces of 30 heavily infected rabbits, all of which died, that two exposed rabbits also died of ascaris pneumonia. The susceptibility of rabbits to serious lung damage from ascaris larvae has also been shown by Ransom and Foster (1920).

The negative autopsy finding following the feeding of 51,000 infective eggs to a three-year-old dog must be contrasted, in spite of the fact that a dose as small as 72 of these eggs produced about 50 lesions in the lungs of a guinea pig, with the experience of Fülleborn (1927) who found that a small young dog fed about 40,000 pig ascaris eggs exhibited a pneumonic process at autopsy 2 $\frac{3}{4}$ days later. He also recovered many ascaris larvae from the lungs.

The observation of insusceptibility, apparently complete, in cats to 5,000, 25,000 and 50,000 infective *A. lumbricoides* eggs parallels the results of Ransom and Foster (1920) in their negative finding in one halfgrown cat "fed many thousands of eggs of *Ascaris suum*."

Thus it is seen that experiments comparing the susceptibility of several common laboratory animals to lung infection with *Ascaris lumbricoides* larvae have revealed differences as well as similarities which are consistent. Clearly, the ability of *A. lumbricoides* to migrate and produce lung lesions in one host can no longer be accepted as a priori evidence that such migration will occur as well, if at all, in other host species. The question may well be raised as to whether the danger of lung migration of aberrant ascaris larvae in human beings really has a sufficient basis of facts.

The use of unknown numbers of eggs in much of the experimental work of the past has not only served to obscure this differential susceptibility of various hosts to lung infection but has also failed to give adequate information on the infectivity of egg cultures. This need no longer be true. Known infective eggs should certainly be a prime prerequisite for any experimentation.

SUMMARY

Following the administration of known numbers of infective eggs, rats have repeatedly been shown to be poorer hosts for the demonstra-

tion of lung lesions due to the migration of *Ascaris lumbricoides* larvae than guinea pigs. Mice were shown to be only slightly more susceptible than rats to lung infection, while chickens were less effective than rats for the demonstration of lung lesions resulting from the migration of *A. lumbricoides* larvae. Rabbits were found to resemble guinea pigs in their susceptibility to lung damage with *A. lumbricoides* larvae. A dog, an opossum and three cats failed to reveal lung lesions following feedings of large numbers of eggs known to be infective for guinea pigs in doses of 100 eggs.

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STUDIES ON STRONGYLOIDES

IV. SEASONAL VARIATION IN THE PRODUCTION OF HETEROGONIC PROGENY BY SINGLY ESTABLISHED *S. RATTI* FROM A HOMOGONICALLY DERIVED LINE

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INTRODUCTION

In those *Strongyloides* infections where both modes of larval development are commonly encountered, there is little constancy in the daily yields of heterogonic, i.e., indirect or free-living adult, in contrast to homogonic, or direct, progeny. This variability has received the comment, among others, of Brumpt (1921), Sandground (1926a and 1926b), Schuurmans-Stekhoven (1928), and Lucker (1934) concerning *Strongyloides papillosus*, *S. ratti*, *S. stercoralis*, and *S. ransomi*, respectively.

In the present study periodic fluctuations in the production of adults of indirect development are reported for single larva infections of *S. ratti*. Under prolonged observation the frequency of occurrence of cultures which are made daily and which yield heterogonic adults increased and decreased over long seasonal cycles. This seasonal variation is distinctive in character, and appears not to have been heretofore recognized in the biology of this parasite.

The demonstration of seasonally changing progeny cycles in *S. ratti* evolved from observations made almost continuously since January, 1935, on 153 parasites, each established in a rat by means of a single homogonic larva. From these 153 infections, 9181 24-hour fecal cultures were obtained which yielded one or more offspring upon isolation. Over 75,000 progeny have been classified according to the mode of larval development which they had pursued.

Materials and Methods

Of the 153 single *S. ratti* infections from which data were obtained, 127 were of a pure line originating from a single homogonic larva which has been maintained by serial passage. Among the remaining 26 single larva infections, seventeen were first generation infections from the same stock strain of *S. ratti* from which the pure-line group was obtained. Five were second generation, and four were third generation infections from these new lines. Differences, if any, between these extra infections and the pure line are slight, and the data for the present study represent the total experience with these infections.

During the early period of study on these single *S. ratti* infections, certain data were obtained from cultures in which the fecal pellets were either crushed or mixed with powdered bone charcoal. These data were excluded from previous analyses (Graham, 1938a and 1938b). While the progeny yields from the 675 daily cultures thus involved are no doubt lower than would have been obtained in uncrushed cultures, the divergence in the relative yields of heterogonic and homogonic progeny between "mixed" and "unmixed" cultures was not sufficiently great to warrant the exclusion of such a large body of data, and they are accordingly included in the present analysis. These 675 culture days were in the months January to May, 1935, and are represented by 15, 178, 404, 29, and 49 cultures, respectively.

Additional details of technical procedure have been presented in previous articles (Graham, 1936, 1938a and 1938b) and prevail similarly for this study. It should be emphasized that the unit item under consideration is the yield of offspring obtained from the fecal output, over 24-hour periods, from a rat harboring a single *S. ratti* parasite. The progeny were concentrated by Baermann isolation of these "fecal collections" after the "daily cultures" had been incubated for 48 or 72 hours. All fecal collections were so made and so examined. In this particular study it is the yield of heterogonic or indirect adult progeny from such "culture days" which has shown the cyclical seasonal fluctuation.

RESULTS

During the first few months in which infections of single *S. ratti* of homogonic origin were observed by daily fecal collections, free-

living adult progeny seemed to appear entirely at random. But in June and July, 1935, there was an increased frequency of culture days with such progeny. This was so marked that the possibility of a seasonally correlated influence was strongly suggested. Almost continuous study since that time has confirmed that suggestion.

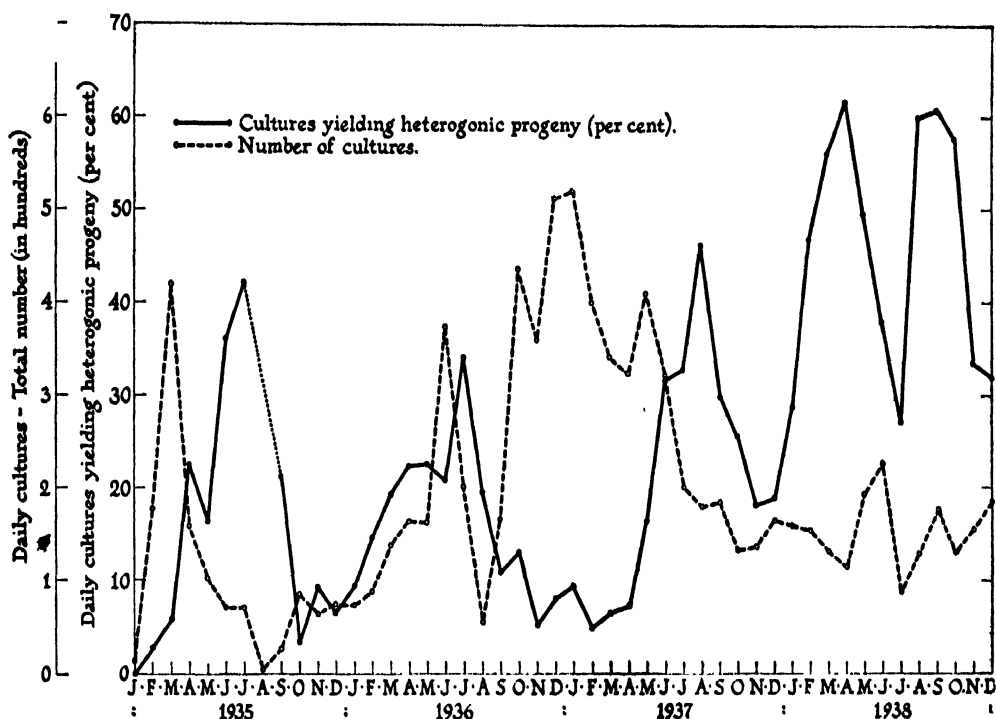


FIGURE 1. Comparison of the monthly distribution of cultures yielding heterogonic progeny, with the total number of cultures containing progeny. The data represent 9181 daily cultures from 153 single, homogonically derived *S. ratti* parasites.

The monthly percentage of culture days with indirect progeny is shown graphically in figure 1, together with a curve indicating the total number of cultures examined. It will be seen that the percentage of daily cultures which yielded adults of indirect development increased from January, 1935, until July, when a seasonal maximum of 42 per cent was reached. Then a period of decline began, and a level of less than 10 per cent was reached in October to January, 1935-36, after which the percentage began to increase in 1936 and attained a maximum in July, at 34 per cent. It then declined to less

than 10 per cent (November to April, 1936-37). In August, 1937, 46 per cent of all cultures yielding one or more offspring contained progeny of indirect development. For 4 months after this 1937 maximum was reached, a characteristic post-summer regression occurred.

For the years 1935 to 1937, the seasonal fluctuations in the percentage of daily cultures yielding indirect adults describe a curve with seemingly good rhythmicity, reaching maxima in July, July and August, respectively, and with intervening minima spaced about equally. During 1938 a somewhat unusual situation has been observed. From December, 1937, the percentage of cultures containing free-living adults increased steadily for 4 months before a recession began. From February to May, 1938, percentages were obtained which were higher than the midsummer maxima of the 3 preceding years. In 1938, the percentage curve was distinctly bimodal and correlated to a considerable degree with locally abnormal climatic conditions.

Consideration of Contingent Factors

1. *Numbers of cultures examined.* The above finding has been analyzed from several different angles. While there has been considerable variation in the number of daily cultures available from month to month which yielded one or more offspring, more than 100 cultures were examined in 36 of the 48 months covered by this study. In only three instances did the number examined drop below 50. It is clear, from the curves shown in figure 1, that there is no relationship between the percentage of cultures with offspring of indirect development and the number of cultures examined in each month. Thus, it can be assumed that the points on the percentage curve are adequately, though not equally well, established.

2. *Age of parasite.* Concerning the age of the parasite as a factor in determining the mode of development followed by the progeny, it has already been shown (Graham, 1938a) that the production of adults of heterogonic development occurs at a fairly uniform rate throughout the entire life of the parasite. This is in marked contrast to the condition seen among the progeny of direct development. These are produced in increasing numbers during the early weeks of the parasite's life. Then the number declines more or less abruptly,

reaching a level where the rate of decline is very low. In some instances this low reproductive rate may continue for more than a year.

3. *Age in relation to season.* In addition to scrutinizing age of the individual *S. ratti* independently of the season of the year, it is necessary to examine parasite age in relation to season. In figure 2 the two curves show the percentage of culture days on which free-living adult progeny were obtained from parasites of two age groups, namely, less than 3 months of age and over 3 months of age. During 1935 and the early part of 1936, only a small percentage of the parasites studied were over 3 months old. Since then, approximately equal numbers of cultures have been obtained from parasites in each age group. The parallelism of the two percentage curves in figure 2 is marked and shows no evidence of age (i.e., older or younger than 3 months) as the factor determining the heterogonic mode of larval development. Other age groupings of the parasites have been explored, and suggest no other interpretation. Whatever may be responsible for the seasonal variation in the frequency with which progeny of heterogonic development are produced it appears definitely to affect young and old *S. ratti* parasites alike.

4. *Fecal environment.* Consideration of the above facts indicates that the seasonal changes in the rate of production of heterogonic progeny must be accepted as a reflection of an actual change in the reproductive performance of the singly established, homogonically derived *S. ratti*, unless the influence of the fecal environment to which the newly-hatched larvae are exposed can be shown to influence their mode of development. While an earlier analysis (Graham, 1938a, p. 231) gave no warrant for assuming any measurable effect of such influence, another conclusive elimination of this possibility was desirable. This was procured from a comparison of the progeny yields obtained from the present homogonic series of infections and a series of single *S. ratti* infections established with larvae of *heterogonic* origin, i.e., infective larval progeny of the free-living bisexual generation. From September, 1937, until July, 1938, these two series were studied in parallel and are thus directly comparable. All infections were handled similarly and subjected to identical technical procedures. They differed only in that the singly established parasites in the one case were of homogonic origin, in the other of heterogonic origin.

The progeny relationships between these two series were markedly

different. The heterogonic series produced *heterogonic* progeny at a rate nearly six times that observed in the homogonic series. This obviously indicated that parasites of different constitutional potentialities were being compared, and that culture conditions, i.e., environmental influences, were not significantly involved as prevailing variables. With the establishment of these facts, the demonstration in the homogonic series of a *distinct seasonal rhythm* in the appearance of adults of indirect development (as found in cultures made daily)

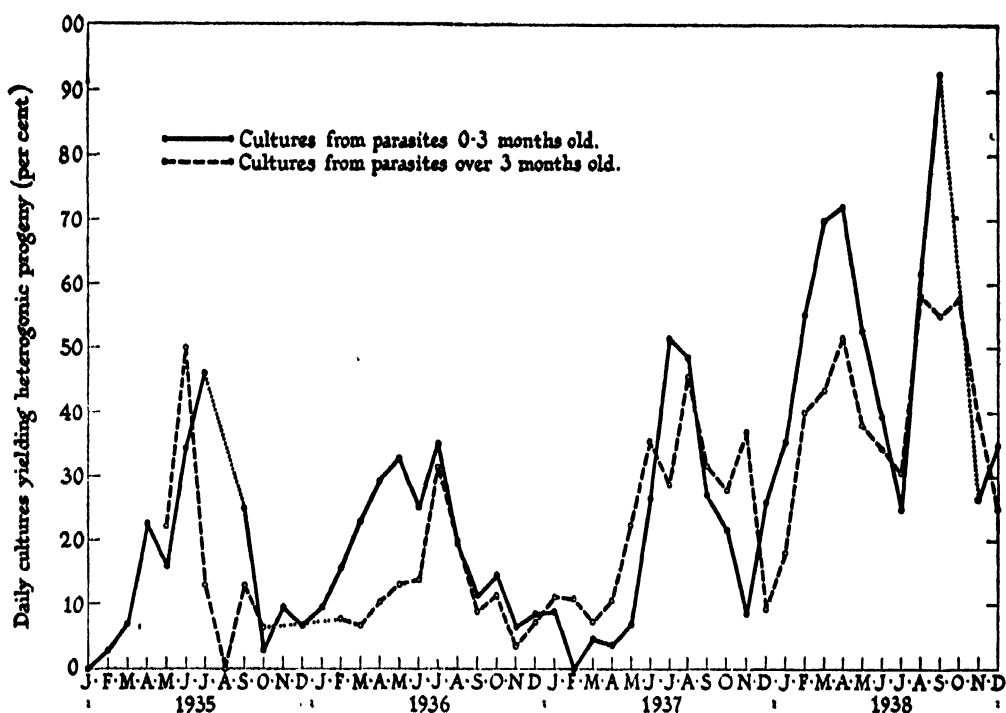


FIGURE 2. The monthly distribution of cultures yielding heterogonic progeny (i.e., the solid line curve of figure 1) subdivided to show the distribution from single parasites of *S. ratti* up to 3 months of age and over 3 months old.

assumes a significant character. This consistent behavior of a homogonic strain through four consecutive annual cycles adds another to the factors involved in determining which of the alternative modes of development the young offspring of *S. ratti* will pursue.

Meteorological Factors

A single *S. ratti* in the intestine of its preferred host species is presumably living under ideal conditions. One is accustomed to

visualize this intestinal environment of the parasite as comparatively stable, tending to induce uniformity in the biological reactions of a parasite. Yet the insignificance of factors considered in the preceding section as influential elements in producing the observed seasonal fluctuations clearly suggests that this intestinal environment is less uniform than one might suppose. What factors, operating with a seasonal periodicity, could induce changes in the environment of the parasite? While it is scarcely possible that essentially epidemiological

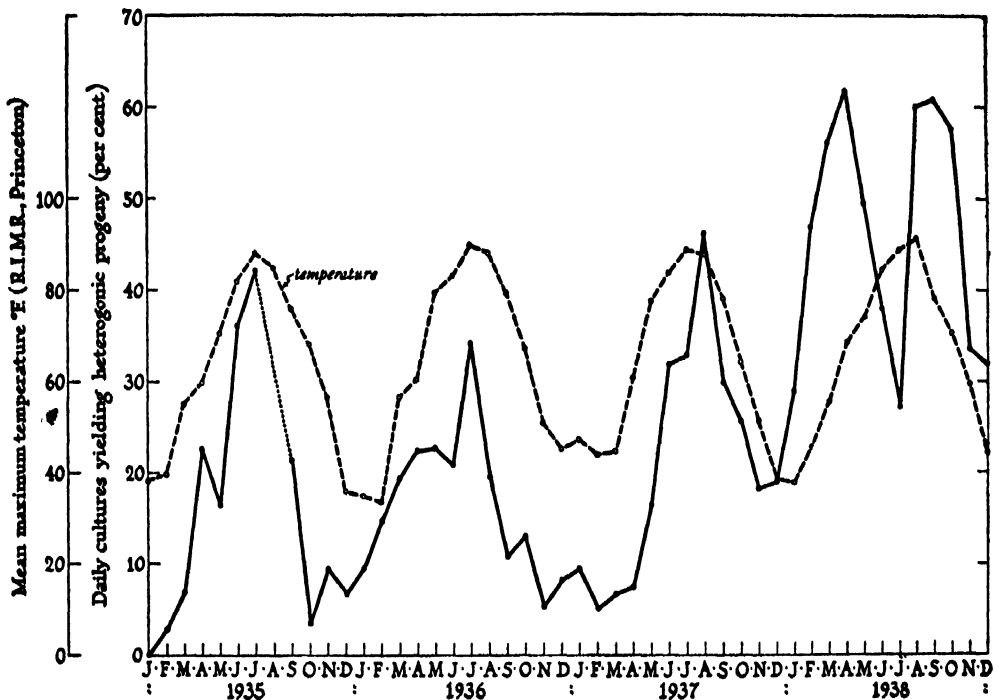


FIGURE 3. The solid line curve of figure 1 (i.e., the monthly distribution of cultures yielding heterogonic progeny) shown in relation to the mean monthly maximum temperature as determined at the institute laboratory, Princeton, N. J.

data can provide a complete answer to this question, certain meteorological factors which fluctuate seasonally immediately suggest themselves.

1. *Temperature.* Among the climatological elements which deserve consideration, temperature is perhaps the most obvious, although humidity, length of day, amount and intensity of light, as well as others, may be involved, either singly or in combination. In figure 3 the curve showing the percentage of culture days on which progeny of

indirect development were obtained is shown with the curve of mean, monthly, maximum temperatures out of doors as regularly read at the institute laboratory. It will be seen that there is a marked correlation over almost the entire period of this study, although it should not be assumed that this correlation necessarily implies causal relationship. (To illustrate such a caution, the monthly percentage of cultures yielding progeny of indirect development is as well correlated with the total hours of sunshine in each month, determined by the U. S. Weather Bureau at Trenton, 12 miles distant, as it is with temperature.)

While there was some fluctuation in the temperatures to which the rat hosts harboring the single *S. ratti* were exposed, the mean seasonal temperatures to which they were exposed did not vary over as wide a range as did the mean, monthly, maximum temperatures out of doors. The daily temperature *range* in the room in which the rats were housed fluctuated from 8° to 20° F, with a most frequent range of 10° to 12°. During summer the predominant temperatures ranged from 80° to 90° F, during winter from 70° to 80° F. Thus the effective mean seasonal temperature change was about 10°. This would not be considered excessive, but even so it did express itself in increased food consumption and increased fecal output by the rats during the colder periods of each year.

2. *Relative humidity.* While the mean, monthly, relative humidities were not as clearly correlated with the frequency of appearance of cultures yielding progeny of heterogonic development as were the mean, monthly, maximum temperatures, several suggestive relationships existed. In the first place, there was a rather definite seasonal cycle which the relative humidity followed. These relative humidities, shown in figure 4, are averages of the monthly mean values determined from readings made daily at 8 A.M., 12 noon, and 8 P.M. by the U. S. Weather Bureau at Trenton. It will be noted that the relative humidity occasionally deviated considerably from the normal. In the years 1935 to 1938 inclusive, the major annual depression occurred in the spring months. A comparison of the relative humidity curve in figure 4 with the superimposed curve showing the monthly percentage of cultures yielding free-living adult progeny reveals that the maximal points on the latter in 1935 to 1937 followed periods of major depression in the former in each year. It is obvious that the

depression of the relative humidity at this particular time of year is a direct result of increasing temperature. The biological result of this change is a marked increase in the cooling power of the animal body, a process which conceivably may lead to profound physiological readjustment.

Although recurring sequences involving relative humidity and the reproductive performance of this homogenically derived strain of

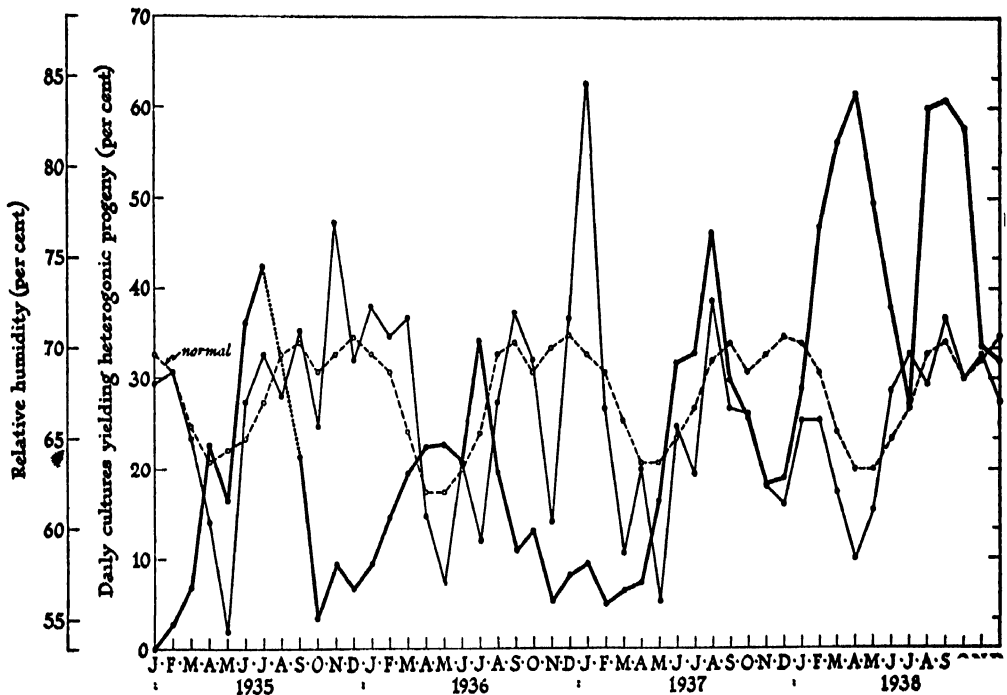


FIGURE 4. The monthly distribution of cultures yielding heterogonic progeny (i.e., the solid line curve of figure 1) shown in relation to relative humidity. The light dotted line and the light solid line represent the normal and the observed relative humidity, respectively.

S. ratti may have no factual connection, it is notable that an atypical increase in the frequency of appearance of cultures containing indirect offspring has occurred since December, 1937. This followed a depression of considerable magnitude in the relative humidity. Moreover, it may be significant that, for a period of 9 consecutive months (September, 1937, to May, 1938, inclusive), the relative humidity was below normal.

While the rise in the frequency curve in the spring of 1938 was

unusual both as to its unseasonableness (as judged by available evidence) and its magnitude, it is worthy of note that the situation prevailing in the comparable period of 1936 may have differed only in magnitude. The tendency for the curve to rise consistently was manifested during the same time of year, viz., from December of the preceding year through April. Climatologically, this period of 1936 was characterized by relative humidities equalling or exceeding the normal, whereas in 1938 they were distinctly subnormal. Although the evidence concerning relative humidity is admittedly inadequate for the drawing of other than tentative conclusions, one fact stands out clearly; that, on *every* occasion on which the frequency of appearance of cultures yielding progeny of heterogonic development rose above 30 per cent, the increased frequency *followed* a few months after a major depression of the relative humidity.

It seems likely that at times of the year when the differential between the animal room temperature and the outdoor temperature was greatest, i.e., in winter, the differential in the relative humidity was likewise at a maximum. Under such conditions the indoor relative humidity would be markedly lower than that prevailing out of doors. Since wet bulb temperature readings were not made in the animal room, it is not possible to present the actual variation of the relative humidity to which the rats were exposed throughout the year. In the absence of critical data obtained under adequately controlled experimental conditions, judgment concerning a possible rôle of relative humidity in producing the observed results must be deferred. Relative humidity has been shown to be an important factor in several biological rôles and may conceivably be involved in the present instance.

DISCUSSION

The observation of a seasonal change in the rate of production of progeny of heterogonic development by single, homogonically derived *S. ratti* introduces a heretofore unappreciated element in the biology of this species. It is clear that whatever seasonally correlated factors activate the cyclical variation in the production of progeny of indirect development must ultimately influence the parasite's reproductive mechanism. It is also patent that these factors must operate through

the medium of the host in order to affect *S. ratti*. This capacity of the host to influence the reproductive performance of the parasite seems appropriately referred to as a cyclical physiological state. It appears likely that these seasonal changes in the rat are induced through the agency of meteorological factors. Such action of climatic factors on the reproductive performance of any species of *Strongyloides* has not previously been suggested, although Kreis (1932) erroneously attributed to Sandground (1925) the statement that "the development is dependent on meteorological conditions." (Cf. Sandground, 1925, p. 240.)

That environment might play a rôle in determining the mode of development followed by *Strongyloides* larvae was given prominence in the hypothesis which Leichtenstern (1899) advanced to account for the apparent (possibly real) preponderance of progeny of indirect development in *S. stercoralis* in cases of tropical origin as compared with infections in temperate regions. His views are perhaps best expressed in his own words: "in der gemässigten Zone die zur Rhabditis [free-living bisexual] generation bestimmten Embryonen ungünstiger Aussenverhältnisse halber zu Grunde gehen, während die direkt transformierten Larven sich erhalten und die Fortpflanzung der Species von Generation zu Generation besorgen." This hypothesis, according to Sandground (1926a), "failed to provide satisfactory explanations for many of the facts," although he agrees with Leichtenstern's (1898) conclusion that the mode of development of the *Strongyloides* larvae was "ein immanente oder prädestinierte Eigenschaft des betreffenden Embryos, d.h. ein Funktion seines Erzeugers." Thus it is clear that neither Leichtenstern nor Sandground was impressed with the possibility of climatic influence on the host as an influencing factor in determining the mode of larval development in *Strongyloides*. However, it is possible that Leichtenstern (1899) actually observed such a phenomenon without recognizing its significance. He observed "dass bei unserem, seit 13 Jahren beobachteten Falle Berlemont, mitunter wochen- und selbst monatelang nur allein die direkte Larvenbildung beobachtet werden konnte. Die Anguillula unseres Berlemont besitzt eben nach zahlreichen Generationen, die auf dem Wege der Selbstinfektion erfolgten, die vorwiegende, ja längere Zeit hindurch oft ausschliessliche Tendenz zur Erzeugung direkt transformierender

Embryonen; dann aber erfolgt gewissermassen als Rückschlag auf die tropischen Ureltern wiederum einmal eine Anguillulageneration, deren Embryonen teilweise auch die Rhabditisgeneration liefern." That the "retrogression" observed by Leichtenstern in "Berlemont" is evidence of changes in the physiological state of the latter seems more likely than that "auto-infection" was involved in the manner suggested. As a matter of fact, "auto-infection" or "hyperinfection" has not yet been acceptably demonstrated with any species of *Strongyloides*.

With the demonstration of constitutionally dissimilar lines of *S. ratti*, there is no reason for questioning further Sandground's belief that the mode of larval development is determined by inherent factors. This point of view has been held by many of the investigators who have studied various species of *Strongyloides* during the past 6 decades. While the evidence adduced in support of the speculations of Leichtenstern (1898), Brumpt (1921), Schuurmans-Stekhoven (1928), and others was for the most part fragmentary, these bits of information have consistently pointed in the direction indicated. On the other hand, no acceptable evidence supporting the view that the mode of larval development in any species of *Strongyloides* is determined in an external environment has yet been reported.

The present study suggests that the rat plays a significant rôle in determining the mode of development followed by the progeny of the homogonic strain of *S. ratti* under consideration. As a result of changes which he observed in *S. stercoralis*, *S. papillosus*, and *S. fülleborni* when they were transferred from their normal to abnormal hosts, Sandground (1926a) concluded that "there is some relation between the host and the parasite which plays a part in determining the constitution of the eggs produced by the latter." Continuing his speculations, he conjectured that the reproductive processes "in the parasitic mother worm are in some way controlled by the environment encountered in the host, perhaps through the metabolism of the parasite." Brumpt (1921) expressed a similar point of view when he stated that "Si nous connaissions mieux les milieux intestinaux des animaux, nous pourrions peut-être approcher de la solution recherchée et expliquer le déterminisme de sexes et de l'hétérogénèse."

The logical corollary to such statements would be that host influence

on the parasite was susceptible to temporal variation. Such variation would, in part, depend on exposure to environmental vicissitudes to which the host was capable of making a physiological response. The labile nature of the reproductive mechanism of the human, sheep, chimpanzee and rat *Strongyloides* has thus had substantial demonstration. Of these, only *S. ratti* has been observed in its normal host species under conditions favorable for the detection of seasonal variation in the host influence on the reproductive process.

The demonstration of a cyclically varying physiological state in the rat in the present instance is due entirely to a peculiarity of *S. ratti*, namely, the faculty of producing progeny of two morphologically distinguishable types. In this connection it is suggested that other worm-host relationships may vary because of climatic influences on the host and that the demonstration of these changes merely awaits the application of suitable procedures to reveal and measure them. In fact, evidence on this point is already at hand. In a recent paper, Kubo (1938) reported studies made at Peiping, China, on two dogs infected with *Dirofilaria immitis* which were "exposed to the influence of atmospheric temperature." He observed that "the total number of microfilariae present in the peripheral blood over the period of a whole day is very much higher in summer than in winter."

It is a matter of interest also to observe that a constitutional difference between two lines of *S. ratti* has been conclusively demonstrated, the lines being of indirect and direct origin, respectively. This fact, taken in conjunction with the demonstration of an environmental factor which operates *through the agency of the host on the parasite and not on the larvae in the fecal cultures*, rephrases the problem of explaining the heterogonic mode of development in *Strongyloides ratti*. Finally, the present studies facilitate the understanding of the speculations, and to a considerable extent, the inconclusive experiments, which have made this problem perennially confusing.

Meteorological Factors in Relation to Certain Biological Phenomena—a Discussion

Concerning the effect of meteorological factors on biological processes, there has been an increasing number of researches and epidemiological investigations reported in recent years which tend

more and more to establish climate or climatological derivatives as dominant elements in physiological reactions of diverse nature. Among these, Kligler (1936) has discussed certain pertinent evidence concerning the influence of climate on susceptibility to enteric infections. Mice infected with *S. enteritidis* and kept at various temperatures and humidities responded differentially, according to Kligler and Olitzki (1931). They concluded that the critical factor appeared to be "not temperature alone nor humidity alone, but the combination of the two."

Gill (1936) reported certain epidemiological features of the malaria epidemic in Ceylon in 1934-35 which, if based on valid premises, are of fundamental significance. As a working hypothesis Gill suggested that "a sharp rise of relative humidity [drought broken by heavy rainfall] during the preëpidemic period is in some way responsible" for the "'epidemic of relapses' amongst apparently healthy human carriers." Two distinct and sudden increases in relative humidity which occurred about 6 months apart were both followed after an interval of about a month by sharp rises in the morbidity curve. Gill attached primary importance to the "change in the relationship of the malaria parasite and the human host, whereby, at the commencement of an epidemic, an 'epidemic of relapses' is precipitated."

The change in the host-parasite relationship in malaria infections (as suggested by Gill) may be compared with the increased frequency with which daily cultures containing progeny of indirect development were procured from single *S. ratti*. As seen in figure 4, each increase in the frequency curve *followed* by a few months some major depression of the relative humidity curve. In this respect then, the seasonally changing progeny cycle of this strain of *S. ratti* resembles the drought epidemic of malarial relapses in Ceylon. The analogy suggests points in common. In both cases it is possible that the sudden increase in humidity may have led to profound physiological readjustment on the part of the host with subsequent changes also resulting in the parasite in each instance. It may be, as Huntington (1938) stated, that the "physiological responses [of rats] to diet, disease, and weather are surprisingly like those of man." That rats may react seasonally in normal physiological processes has been shown by King (1927) who has reported a marked seasonal *variation in the fertility*

of an albino rat colony, which breeds the year around. She stated that "Temperature is seemingly the one environmental factor that could have altered the reproductive rhythm." Recognizing, however, that other possibilities existed she suggested that where "temperature changes are slight, as in tropical regions, humidity may be the factor that largely determines sexual activity." The extent to which such cyclic reproductive changes in the rat, together with presumably attendant endocrine changes, may be correlated with the change in progeny types in *S. rattii* is unknown.

A statistical study of 17,116 obstetrical cases in Calcutta was made by Mitra and Ghosh (1938) with respect to the incidence of eclampsia. They concluded, on the basis of a fair negative correlation with maximum temperature that "seasonal influence" had "some definite relationship." The correlation with relative humidity was poor, and they stated that "humidity plays a very minor rôle." Their graphic and tabular data, however, clearly show that the curve of "incidence of eclampsia" lags one or more months behind the relative humidity curve. This is significant, and permits an interpretation quite different from that of the authors, who relied exclusively on the coefficient of correlation. As a matter of fact the lag of the "incidence" curve behind the relative humidity curve suggests that this lagging is a phenomenon similar in some physiologic aspects to the lag of malaria morbidity after an increase in relative humidity, as noted by Gill (1936) in the Ceylon epidemic, and the relationship which possibly exists between relative humidity and the production of progeny of indirect development by *S. rattii* as noted in this paper.

Another instance wherein a biological response, apparently "conditioned by the climate and the weather," is manifested after a time lag has been reported by Mills (1938). He has shown graphically that the curve of "case onsets" of acute rheumatic fever at a Cincinnati hospital lags about one month behind the curve of "storminess," i.e., the coefficients of temperature variability.

Other seasonal variations in mammals, aside from the well-known annual cycles in reproduction, have been reported. Lillie, Dyer, Armstrong, and Pasternack (1937) observed that the intensity of the brain reaction, both of mice following inoculation with virus of St. Louis encephalitis and of guinea pigs with endemic typhus, varied

with season. The brain reactions observed were milder during the summer than in winter, and the authors considered the variations to be "a direct environmental temperature effect." Likewise, Pearce, Brown, and Van Allen (1924) obtained results with a transplanted neoplasm in rabbits which "emphasize the occurrence of two periods of maximum malignancy—one in the spring and the other in the fall—." They stated that the "peculiar coincidence of effects is strongly suggestive of the action of some influence commonly referred to as meteorological." Stephens et al., (1918), using quinine in treating human cases infected with "simple tertian malaria," observed that "a very small percentage of cures is obtained in the winter and spring and a comparatively high percentage in the summer and autumn." They stated that, to their knowledge, "the only meteorological factors that are in any way correlated with the seasonal variation in the percentage of cures obtained by us are the variations in the mean temperature, and prevalence of the east wind."

These few references give some indication of the variety of associations or conditions wherein changes in the physiological state of animals are presumably induced through the agency of meteorological factors. They convey the obvious suggestion that here is a variable, frequently ignored in experimental and epidemiological investigations, which may exert considerable influence.

SUMMARY

1. The frequency with which the daily yield of offspring from single *S. ratti* included adults of heterogonic development has been observed to fluctuate over long seasonal cycles.
2. In the years 1935 to 1938 inclusive, the singly established parasites produced maximal numbers of heterogonic offspring during the spring and summer months.
3. A correlation existed between the monthly percentages of culture days on which adults of indirect development were obtained and the monthly mean maximum temperature.
4. On five occasions, once each in 1935, 1936, and 1937, and twice in 1938, the monthly percentage of cultures containing free-living adult progeny increased to maxima ranging from 34 to 62 per cent.

On each occasion, the maximal values were reached from 2 to 4 months after a major depression in relative humidity.

5. These data suggest that the rat responds physiologically to changing meteorological conditions and that the altered environment thus produced for *S. ratti* leads to changes in the relative frequency with which progeny of heterogonic development are produced. This modified reproductive activity of *S. ratti* is viewed as being mediated through the agency of the host and not as the result of a changed environment encountered by the young larvae outside the host's body.

6. All of the evidence obtained from these studies indicates that the mode of larval development followed by the progeny of *S. ratti* is determined prior to oviposition.

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INTRACELLULAR MICROÖRGANISM-LIKE BODIES IN THE TICK *DERMACENTOR VARIABILIS* SAY

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Pinkerton and Hass (1937), in a study of the microörganisms naturally occurring in *Dermacentor variabilis* collected in Massachusetts, noted, in the ovaries of all the ticks studied, numerous intracellular coccoid bodies staining dark blue with Giemsa and often appearing ring-shaped. Such bodies were also occasionally found in the lining cells of the alimentary tract, but not in any other tissue. Although many other kinds of microörganisms were found in some of the ticks, only these coccoid bodies occurred in all of the ticks.

The development of these bodies during the life cycle of *D. variabilis* forms the subject of the present communication.

Material and Methods

Adult ticks were collected near Princeton, New Jersey, and Woods Hole, Massachusetts, during the summer of 1937. From these, a number of laboratory-reared lines were started. All the stages were fed on guinea pigs and were kept during their fasting periods in tubes held over moist sand in an incubator at 25° C. Material for study was taken only from those lines which during two laboratory generations showed no microörganism-like bodies other than the coccoid bodies.

Entire ticks were fixed in Zenker or in Carnoy-Lebrun. After fixation the ticks were washed in 2 changes of water and were then stored for several weeks to several months in 70 per cent alcohol. They were prepared for sectioning by the method recommended by Slifer and King (1933) for grasshopper eggs. The ticks were placed in 70 per cent alcohol with iodine, then in 70 per cent alcohol, 80 per cent alcohol with 4 per cent phenol, 95 per cent alcohol, aniline, xylene,

and xylene-paraffin. They were embedded in paraffin in the usual way. Enough of the paraffin block was then cut away to expose the tissue, and the block was soaked overnight in water. It was finally sectioned in the ordinary manner. By this method, good sections were obtained of larvae, engorged larvae, nymphs and recently molted adult females, as well as fair sections of engorged nymphs and of a partially engorged female. Entire males could not be sectioned satisfactorily.

Eggs of known age were first fixed in hot Carnoy-Lebrun and were then partially embedded in paraffin in the well of a culture slide. The eggs were covered with Ringer's solution. Each egg was removed from its shell under a dissecting microscope, using needles ground to a fine point. The eggs were further treated in the same manner as entire ticks.

The sectioning of malpighian tubes and ovaries dissected out from adult ticks presented no difficulties.

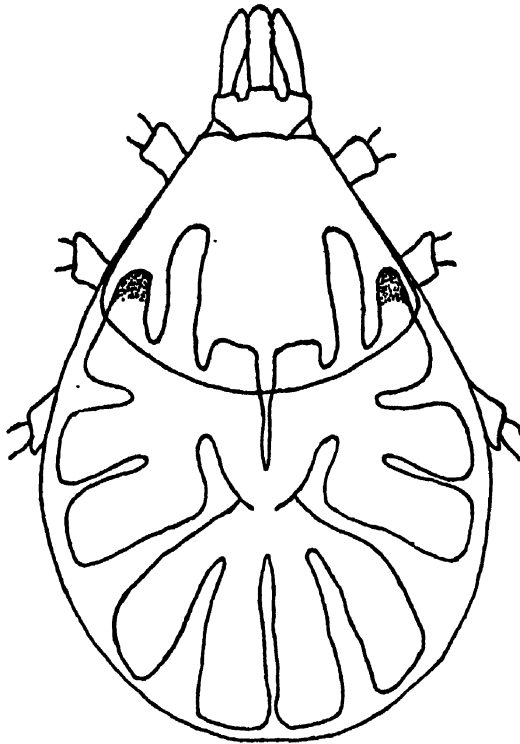
All the sections were stained by Wolbach's (1919) Giemsa method. Complete serial sections of a large number of larvae and nymphs and of some eggs and adults were studied. Smears were also prepared and were stained both with Giemsa and with Gram's stain.

Observations

In *D. variabilis* larvae which were ready to feed, and hence at least 10 days old, masses of the very distinct dark purplish-blue coccoid bodies occurred only in the cells forming the anterior tip of the two more lateral anterior diverticula of the alimentary tract (Text-Fig. 1, Plate I, Fig. 1). The bodies varied in diameter from 0.4 to 0.8 μ . Many of them were clearly ring-shaped, the center of the ring appearing a pale pink. Some larvae apparently had larger numbers of the bodies than did others.

The coccoid bodies were present in engorged larvae in the same position as in the unfed larvae. In newly molted nymphs (Plate I, Fig. 2) and also in engorged nymphs they were still restricted to the anterior portions of the two more lateral anterior diverticula of the alimentary tract. Figures suggesting division stages of the bodies were noted in an engorged nymph fixed three days after dropping from its host. At this stage of development the coccoid bodies were

still confined to the anterior diverticula, but in freshly molted adult male and female ticks the bodies were numerous in the basal portions of the cells of all parts of the alimentary tract. In the adults they were slightly smaller than in larvae and nymphs (Plate I, Fig. 3). This fact, coupled with their great increase in number, suggests that rapid multiplication of the bodies occurred during the latter part of the quiescent pre-adult stage. One series of sections through a



TEXT-FIG. 1. Diagrammatic dorsal view of larva of *D. variabilis*. The portions of the alimentary tract containing coccoid bodies are shaded.

female, fixed on the 4th day after attachment to a guinea pig and already noticeably enlarged, showed numerous typical coccoid bodies scattered through the basal portions of the alimentary tract cells. The bodies were also found in sections of ovaries removed from egg-laying females about 3 weeks after engorgement. They were not found in any tissue other than the ovaries and the alimentary tract, thus confirming the observations of Pinkerton and Hass (1937).

The coccoid structures were difficult to find in the developing egg of *D. variabilis*. Enough stages were seen, however, to indicate the probable developmental cycle. When the embryos were 11–12 days old, typical coccoid bodies (Plate I, Fig. 4) were present in yolk cells located near the center of the yolk mass. At this stage, the ganglion of the tick embryo existed only as a ventral thickening of ectoderm, and the dorsoventral muscles had not yet appeared. When the embryos were 14–17 days old, the ganglion, dorsoventral muscles, and rectal sac were definitely formed. The coccoid bodies were still present only in the center of the yolk mass, just above the posterior tip of the ganglion. This was also their location when the tick embryos were 19–21 days old and when they were 23–25 days old and about ready to hatch. In very recently hatched larvae, in which the alimentary tract had not yet formed, no coccoid bodies could be found in the anterior dorso-lateral regions of the body. But in slightly older larvae, having a formed alimentary tract, coccoid bodies were present in the anterior tips of the lateral anterior diverticula, just as in larvae ready to feed.

The morphology of the coccoid bodies is adequately represented by the photomicrographs of Plate I. It is noteworthy that throughout the life cycle of the tick they did not vary greatly in size or appearance. With Giemsa, they stained a dark purplish blue, usually with a more or less distinct pale pink center. In the embryos, they were somewhat more lightly stained. In smears made from larvae and adult females they stained Gram positive.

Very similar bodies were found in sections of four larvae of *D. andersoni** (Plate I, Fig. 5). Here again, the ring-shaped dark purplish blue bodies were present only in the cells of the lateral anterior diverticula of the alimentary tract. They differed from the bodies in *D. variabilis* larvae only in being smaller ($0.3\text{--}0.5\ \mu$) and slightly more numerous, and in extending farther posteriorly. In sections of a few larvae of *Haemaphysalis leporis-palustris* no structures suggesting microorganisms were found.

* These larvae were descendants of adult ticks which Dr. R. R. Parker kindly sent me.

DISCUSSION

The observations reported in the preceding section indicate that the coccoid bodies of *D. variabilis* pass through the following developmental cycle. They are present in the ovaries of adult females, whence they infect the eggs. During embryonic development they come to lie in the center of the yolk mass and they are still in this location at the time of hatching. When the alimentary tract becomes developed they appear in the cells of the lateral anterior diverticula. Here they remain, with possibly a slight increase in numbers, during the larval, engorged larval, nymphal, and early engorged nymphal stages. During the pre-adult stage they multiply and invade the epithelial cells of the entire alimentary tract. How they get into the ovary, and how they get from the center of the yolk mass to the anterior diverticula of the alimentary tract are two problems concerning which nothing is known.

The size and staining reactions of the coccoid bodies, their remarkable uniformity in appearance in all the stages of the tick, and their presence in a certain region of the yolk of the developing egg as well as in the alimentary tract and in the ovaries indicate that they are probably microorganisms rather than food storage or secretion granules. One would hardly expect the identical type of cytoplasmic granule to occur in a limited portion of the alimentary tract, in the ovaries, and in the center of the yolk mass of the embryo. They may be distinguished at a glance from the small dark brown granules resulting from the digestion of hemoglobin, as well as from the large, pink-stained food vacuoles which occur in the alimentary tract cells of fed ticks. Their intracellular habitat, their occurrence in all ticks of the species *D. variabilis* so far examined, and their hereditary transmission place them among the so-called intracellular symbionts (Buchner, 1930; Glaser, 1930).

The occurrence of non-pathogenic apparently hereditarily transmitted microorganisms in ticks was first described in some detail by Cowdry (1925). He found extremely pleomorphic Gram negative bodies in the malpighian tubes and ovaries of 16 species of ticks, among them *D. variabilis*. It has been impossible to confirm his

results so far as this species is concerned. A special search of the malpighian tubes in a large number of serial sections of larvae and nymphs and in smears and sections from adult ticks has revealed nothing suggesting a microörganism, except in a few adults in which uniform rickettsiae-like bodies were noted. These results are in complete agreement with those of Pinkerton and Hass (1937), who found nonpathogenic rickettsiae in the malpighian tubes of only 3 out of 20 ticks examined. It is noteworthy that the photomicrographs given by Cowdry, especially his Fig. 6, which were taken from smears of whole larvae, show structures strongly suggesting the coccoid bodies, but no such bodies are to be found in his drawings made from sections through the malpighian tubes. The extreme pleomorphism figured by Cowdry suggests that he may have been dealing with different structures, some of them microörganisms and some not, rather than with different forms of a single microörganism.

Mudrow (1932) has given an extensive description of the intracellular symbionts of *Rhipicephalus sanguineus*, *Dermacentor reticulatus* and *Boophilus annulatus*, as well as some notes on the occurrence of symbionts in other species of ticks. The organisms were found in the malpighian tubes, ovaries, and eggs. They bear little resemblance to the coccoid bodies of *D. variabilis*. Probably different species of ticks have different symbionts. Pinkerton and Hass (1937), however, found typical coccoid bodies in the ovaries of *Rhipicephalus sanguineus*, whereas according to Mudrow, the organisms of *R. sanguineus* are straight or slightly curved rods.

The most recent work concerned with cytoplasmic microörganism-like inclusion bodies in ticks is that of Gregson (1938). He states that in the gut epithelium of the engorging *D. andersoni*, three types of inclusion bodies may be seen: purple coccoid granules (probably identical with the bodies described in the present paper), oval brown particles, and transparent globules. He then proceeds to deal only with the last mentioned type. These globules are not present in unfed larvae, nymphs, or adults. They appear in the alimentary tract soon after the tick begins to feed, and, as engorgement proceeds, they become progressively larger and more numerous. They are located chiefly at the distal ends of the cells and are eosinophilic. As the globules enlarge, the cells themselves also enlarge. These

properties, as well as the photomicrographs published by Gregson, fit perfectly the food vacuoles of ticks. Roesler (1934) has made a thorough study of the process of digestion in several species of ticks. The ingested blood is rapidly hemolyzed in the lumen of the gut, but all subsequent stages of digestion occur intracellularly. The hemolyzed blood is taken up by the alimentary tract cells and appears as globular food vacuoles in the distal portions of the cells. The food vacuoles are acidophilic and increase in number and size as engorgement proceeds. They are naturally present in small numbers or not at all in unfed ticks. As intracellular digestion proceeds, masses of dark brown excretory granules are formed. All these structures have been seen in the course of the study of the coccoid bodies. The inclusion bodies in phagocytic cells described by Gregson have also been seen. Their appearance suggests that they are food storage or secretion droplets.

SUMMARY

Small coccoid bodies, probably microorganisms, have been found in the epithelial cells of the anterior tip of the more lateral anterior diverticula of the alimentary tract in unfed and engorged larvae and nymphs of *Dermacentor variabilis*. These bodies are present throughout the lining cells of the alimentary tract of adult ticks, as well as in the ovaries and eggs. During embryonic development they lie in the center of the yolk mass, where they are still to be found at the time of hatching. The coccoid bodies were present in all ticks of this species examined. Bodies almost exactly like those of *D. variabilis*, and present in the same portion of the alimentary tract, were found in larvae of *D. andersoni*.

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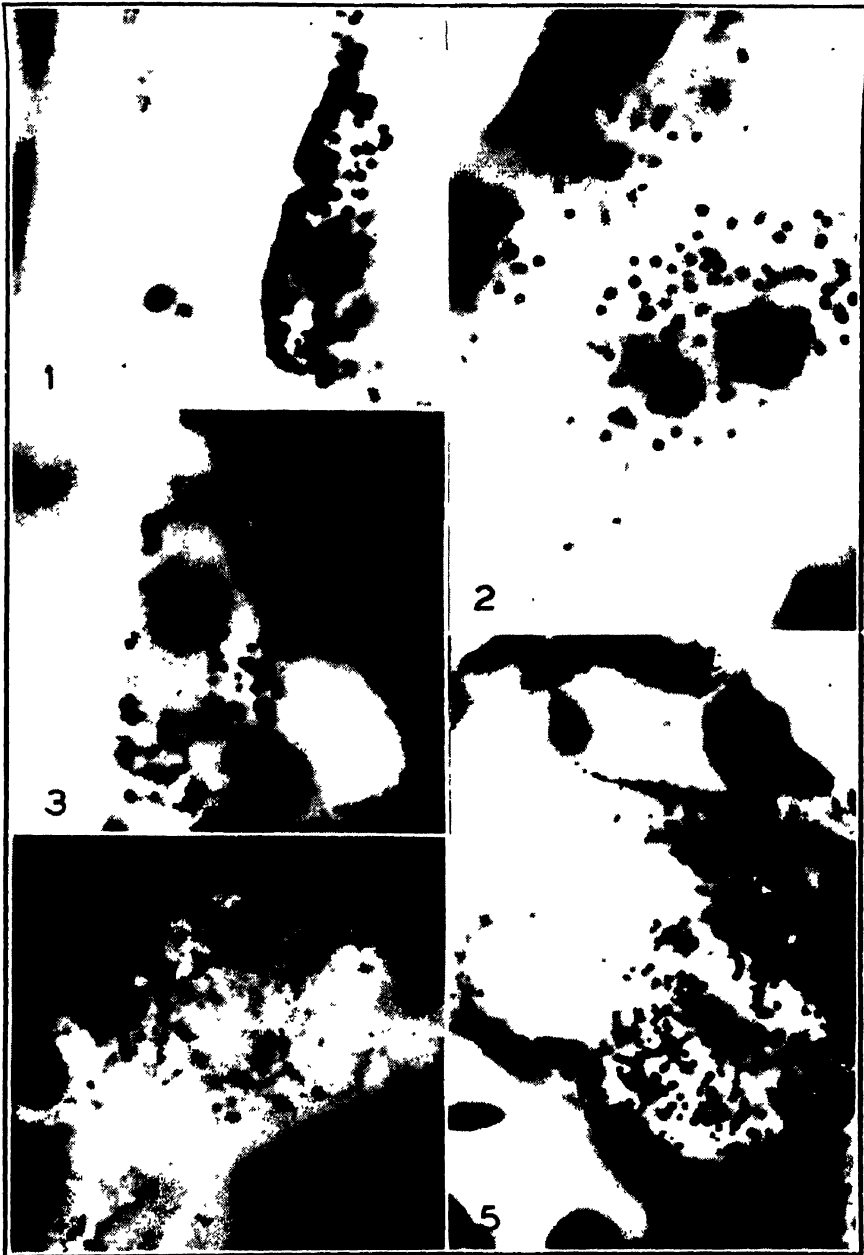


PLATE I

The coccoid bodies in:

- FIG. 1. Larva of *D. variabilis*;
- FIG. 2. Nymph of *D. variabilis*;
- FIG. 3. Adult *D. variabilis*;
- FIG. 4. 11 to 12 day old embryo of *D. variabilis*;
- FIG. 5. Larva of *D. andersoni*.

THE BEHAVIOR OF POX VIRUSES IN THE RESPIRATORY TRACT

II. THE RESPONSE OF MICE TO THE NASAL INSTILLATION OF VARIOLA VIRUS

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PLATES 8 AND 9

(Received for publication, May 19, 1939)

Variola virus introduced cutaneously in the monkey establishes itself in the skin where it produces a characteristic vesicular reaction and apparently retains its identity. Other animals which are highly susceptible to vaccinia, as the calf and the rabbit, are peculiarly refractory to variola. In the absence of an initial reaction it is claimed that successive passages in these animals, either cutaneously or intratesticularly, may eventually be followed by vesiculation. The virus recovered in this event, however, has invariably been vaccinia and not variola. The literature on this so called transformation of variola to vaccinia has recently been reviewed by Horgan (1) who also contributed an example of such a change following testicular passage in the rabbit. The experimental observations on variola in animals have been chiefly confined to these two loci, namely, the skin and testis. In view of the rapid multiplication which vaccinia virus can undergo when implanted on the unbroken nasal mucosa of the white mouse, it seemed of interest to inquire into the behavior of variola virus in the same location (2).

Source of the Virus

The virus used in the following experiments was isolated from crusts¹ obtained from a case of smallpox in a 13 year old boy, a resident of Minnesota.

The history of this case is as follows: Symptoms appeared on Jan. 18, 1938, malaise, headache, nausea, vomiting, and backache, and were followed on Jan. 23 by a macular eruption. Crusts were collected on about Feb. 5 and were shipped to us shortly after in 50 per cent glycerin.

¹ We are pleased to acknowledge the cooperation of Dr. A. J. Chesley, Executive Officer of the Minnesota Department of Health, and of Dr. R. B. J. Shoch, Health Officer of Saint Paul, in arranging for the collection and shipment of the smallpox material, and express our indebtedness to them.

Cultivation of the Virus in Embryonated Eggs

A virus similar in characteristics to the known strains of variola was readily obtained from the glycerinated crusts by transfer to embryonated eggs. The first isolation was made on March 23, 1938, approximately 45 days after collection of the scabs. With the exception of several weeks during the summer when it was maintained in a dried state, the virus has been transferred once or twice a week, 85 passages having been completed. Development of the virus in the egg is characterized by the appearance of focal areas of cellular proliferation and necrosis in the chorioallantoic membrane. If numerous, these areas coalesce forming a solid mass of seminecrotic tissue, sharply demarcated from the normal portions of the membrane. The embryo is not affected.

We have found only two references in the literature to the cultivation of variola virus by the egg technique: one reported by Torres and Teixeira (3) in 1935; the other by Lazarus, Eddie, and Meyer (4) in 1937. Hence it seems desirable to report our own observations in some detail.

One small crust was washed in water, finely ground, and suspended in 2 cc. of saline. 3 embryonated, 10-day, hen eggs were inoculated with small unmeasured amounts of the suspension and incubated at 37°C. The method of Burnet (5) was used, withdrawing the chorioallantoic membrane from the shell membrane and creating a new air sac thereby. Membranes removed on the 2nd and 3rd days from these eggs were normal, but on the 4th day a central cluster of 10 small white foci was observed in the membrane of the remaining egg. This portion of the membrane was removed, suspended in saline after grinding, and inoculated as before. One of the 2nd passage eggs showed focal areas of reaction in the membrane on the 3rd day.

The virus was successfully maintained thereafter in embryonated eggs by inoculation with small amounts of approximately 10 per cent membrane suspensions in saline. To date 85 passages have been made, in most of which a membrane reaction was apparent on the 3rd day after inoculation. Up to the 50th transfer the severity of the reaction fluctuated considerably in eggs inoculated with any one suspension. Thus, in 13 of the 25 passages made prior to the 50th transfer, the number of foci in the membranes of paired eggs varied from few to innumerable. From the 50th passage on the membrane reaction was more uniformly severe.

In inoculated eggs opened on the 3rd day it was customary to find a thickened and heavy membrane with a large oval area, up to 4×3 cm., studded with minute white nodules either discrete or coalesced into small groups. Congestion and hemorrhage were rarely conspicuous. On continued inoculation these focal areas tended to merge forming a solid plateau of seminecrotic tissue. Unless bacterial contaminants were accidentally introduced the embryo was invariably active. On two occasions, however, virus was recovered from washed and ground embryos on subinoculation. If incubation was continued the embryos developed normally, up to the point of hatching, and retrogressive changes were apparent in the involved membrane. Virus was not recovered from the membrane of one egg removed on the 10th day.

Elementary bodies were usually demonstrable in membrane films by the silver method of Morosow. They were rarely as numerous, however, as in membranes from eggs inoculated with vaccinia virus but were indistinguishable otherwise.

One titration was made using a membrane removed on the 4th day from a 15th passage egg. The membrane was weighed, ground, and diluted serially so that the concentration inoculated (0.05 cc.) varied by intervals of 10 beginning with 10^{-2} . A reaction was obtained with all dilutions through 10^{-7} ; of the 2 eggs inoculated with this dilution, the membrane of one showed 6 foci on the 3rd day and the other 12. Sub-inoculation from these 2 membranes was attended by a moderate reaction.

Cutaneous Inoculation of the Virus in Monkeys

Two *Macacus rhesus* monkeys were inoculated into the scarified skin with suspensions of membranes from the 13th and 40th egg passages of the virus. Both animals showed a typical eruption, papules appearing on the 5th day after inoculation. After the local reaction had subsided both animals were tested for susceptibility to variola and vaccinia.

The hair over the sides was removed with an electric clipper, a criss-cross of scratches made in the skin, and the virus suspensions rubbed into the abraded areas with a glass rod. The first monkey received a single dilution spread over a wide area, approximately 0.05 cc. of a 10 per cent suspension being used. Four dilutions, 10^{-2} through 10^{-5} , were inoculated into small squares in the skin of the 2nd monkey. The volume of inoculum, 0.02 cc., was included in the dilution figure. The inoculated animals were held under strict quarantine in an isolation unit and examined daily.

The monkey inoculated with a single dilution of variola virus showed small papules along the lines of scarification on the 5th day. These nodules increased in size and number, reaching a maximum of 20 with a diameter of 5 mm. on the 12th day. At this time some of the nodules were discrete, others confluent. Most of them showed an inconspicuous dirty white tip surmounting a red base. Retrogressive changes with scab formation set in promptly and by the 20th day the area was flat. The virus was active through a dilution of 10^{-4} as indicated by the reaction in the 2nd monkey. Papules were again visible on the 5th day but reached a peak earlier, on the 8th day. At this time the 10^{-2} area showed confluent nodules; the 10^{-3} area, coalesced and discrete ones; the 10^{-4} area, 8 discrete nodules; and the 10^{-5} area, no reaction. Retrogressive changes began on the 9th day and progressed rapidly.

Six weeks after the primary inoculation both monkeys were retested with variola virus and also vaccinia virus, 10^{-2} and 10^{-3} dilutions being introduced cutaneously. There was no reaction to the variola virus in either dilution, indicative of a fairly complete immunity. The vaccinia virus, however, produced a reaction in both animals. The first monkey showed discrete papules after 3 days, on the area which received the 10^{-2} dilution. The 10^{-3} dilution was inactive. Both dilutions produced a reaction in the 2nd monkey, confluent nodules appearing on the 2nd day.

Cutaneous and Testicular Inoculation of the Virus in Rabbits

Four rabbits were inoculated cutaneously, following scarification, with 0.02 to 0.05 cc. of 10 per cent membrane suspensions prepared from the 10th, 13th (2 rabbits), and 34th

egg passages, respectively, and were kept under observation for 3 to 6 weeks. Aside from a transient erythema along the lines of scarification there was no visible reaction. During the 6th week the rabbit which had received the 34th passage suspension was inoculated cutaneously with vaccinia virus diluted 10^{-3} through 10^{-8} . All of the virus dilutions through 10^{-6} gave rise to typical vaccinal reactions, indicative of normal susceptibility.

One series of testicular passages was also carried out in rabbits. The first animal was injected intratesticularly with 0.25 cc. of a 10 per cent membrane suspension from the 34th egg passage. On the 4th day the skin of the scrotum was somewhat thickened and congested at the site of inoculation. The testis was removed on the 5th day and was normal macroscopically and microscopically. Portions of the removed testis and the thickened skin of the scrotum were ground, suspended in several cc. of saline, and 0.25 cc. injected into a second rabbit. 4 subsequent testicular passages were made at intervals of 5 to 7 days. The scrotum and testis showed no visible nor histologic indication of involvement in any rabbit save the first. The rabbits injected intratesticularly with the 3rd, 4th, and 5th passage suspensions were also inoculated cutaneously with the same material. Aside from the initial trauma there was no response. 4 weeks after inoculation the 6th rabbit was tested with vaccinia virus to which it reacted with all dilutions through 10^{-6} .

Cutaneous Inoculation of the Virus in Mice

Five mice were inoculated cutaneously with a membrane suspension from the 64th egg passage. The skin over the abdomen was shaved, scarified, and approximately 0.02 cc. of the suspension rubbed into the abraded area. There was no specific response to the inoculated virus.

Nasal Instillation of the Virus in Mice

Young mice in groups of 5 each were inoculated intranasally with the original suspension of smallpox crusts and with suspensions of the 3rd, 10th, 13th, and 34th egg membrane passages. Titration of the virus content of the several membranes was not made, but judged from the severity of the reaction in the membrane and the number of elementary bodies the concentration was high.

In these and the subsequent experiments white mice weighing 15 to 20 gm. were well etherized and their muzzles were dipped in the test suspension. By this method of administration between 0.05 and 0.1 cc. of fluid is taken up on inhalation. Unless otherwise specified approximately 10 per cent saline suspensions of ground egg membranes were used.

The disposal of the inoculated mice varied from group to group: some were autopsied on the 2nd to the 10th day after inoculation; others were held under observation for a month or longer and injected intranasally with vaccinia virus.

None of the mice injected with variola virus showed either a local or a general response. They gained weight normally and retained their original sleek appearance, quite in contrast to the appearance of mice similarly injected with vaccinia. The nasal passages

were invariably normal at autopsy and elementary bodies were not demonstrable in films. The lungs showed no obvious pathological changes on macroscopic examination. There was some indication that the mice held for a month and then injected with vaccinia were less susceptible than normal individuals, but the observations were too few to be conclusive.

Additional nasal injections were not made in mice until the virus had been carried through 64 egg passages. At this time mice were injected with membrane suspensions from 3 consecutive transfers and killed at daily intervals thereafter to determine whether virus was present in the blood. 2 to 3 mice were bled from the heart under ether on the 1st through the 5th day after nasal instillation of the virus and approximately 0.05 cc. of a 1:10 dilution of blood in saline solution inoculated into 2 embryonated eggs. There was no indication that variola virus was present in the blood in amounts detectable by the egg technique during this period. With the exception of 2 contaminated eggs the membranes were invariably normal when removed on the 3rd or 4th day after inoculation.

While this work was in progress the lungs of 3 mice, killed on the 4th day, were examined by lower power microscopy, using a binocular dissecting microscope which magnified 7 diameters. We had found somewhat earlier in studying a disease of guinea pigs that low power magnification brought out pathological changes in the lung which were not clearly defined by visual inspection. Each specimen showed small but unmistakable areas of consolidation in one or more lobes. A suspension was made from one involved lobe and inoculated into an embryonated egg which was opened on the 3rd day. The embryo was active but the membrane was studded with a fair number of discrete foci.

Additional lung cultures were made from 48 mice injected intranasally with membrane suspensions from the 65th through the 82nd egg passages of the virus and killed at varying intervals through the 7th day.

The first isolations were made from etherized mice killed by decapitation. The lungs of mice killed in this way often showed congestion and hemorrhage which interfered seriously with microscopic examination. Later, the mice were deeply etherized and the lungs removed aseptically with practically no attendant trauma. The separated lobes, in a sterile Petri dish, were examined microscopically under a dissecting microscope at a magnification of 7 diameters. With mice killed on the 1st through the 5th day a suspension was made from one lobe, usually the left, by grinding in the presence of 1 to 2 cc. of saline. All 5 lobes were used in the preparation of lung suspensions from mice killed on the 7th day. Small unmeasured amounts of each suspension were inoculated into 3, 10-day embryonated eggs which were incubated at 37°C. and opened on the 3rd or 4th day.

The results of these examinations are summarized in Table I. 36 of the 48 mice were killed on the 1st through the 5th day after injection. Variola virus was demonstrable in the egg cultures from all of these mice. Three

isolations of the virus were made from the 12 animals that were killed on the 7th day.

Comparatively few bacterial contaminations were encountered in the inoculated eggs even when the entire lung was used. In no case was the embryo affected in the absence of bacteria. The reaction in the membranes of eggs inoculated with suspensions of lungs removed on the 1st through the 5th day varied appreciably from group to group, according to the passage, and also within the different groups. The reaction was more commonly like that in eggs inoculated with a moderate concentration of the virus, characterized by a central oval area studded with discrete foci or small groups of coalesced ones. Short lines or streaks as illustrated in Fig. 4 were frequently seen. Elementary bodies were usually demonstrable in films. In some membranes, however, the reaction

TABLE I
The Examination of Lungs from Mice Injected Intranasally with Variola Virus

Time between injection and examination	Number of mice examined	Number of virus isolations from the lung	Number of lungs with pathological changes
<i>days</i>			
1	11	11	4
4	11	11	8
5	14	14	13
7	12	3	12

was limited to widely separated discrete foci, characteristic of a very low concentration of virus. Sparsely distributed foci, rarely more than 10 in number, were also present in the membranes of the 3 groups of eggs inoculated with 7-day lung suspensions.

Two virus titrations were made in embryonated eggs using whole lung removed 1 hour after nasal instillation and again after 4 days. The end-point was the same for both time intervals in each determination, the limiting dilution being 10^{-6} .

The mice in the above experiment were injected with the 72nd and the 77th egg passages of the virus, respectively. The lungs were weighed immediately after removal, ground, and 20 per cent saline suspensions made. Serial dilutions varying by intervals of 10 were set up and 2, 10-day eggs inoculated with 0.5 cc. of each dilution, the final concentrations ranging from 10^{-3} through 10^{-7} . The inoculated eggs were opened on the 3rd day.

To determine whether any change was detectable in the activity of the virus as the result of its residence in the lung of the mouse, a membrane, showing some 50 foci after inoculation from a lung removed on the 5th day, was carried through 2 successive passages in eggs and then injected cutaneously in a rabbit. In the egg there was no departure from the reaction

characteristic of the long continued passage series. A large area of confluent foci was present in each membrane on the 4th day with no involvement of the embryo. A 10 per cent suspension of the 2nd passage membrane on cutaneous inoculation in a rabbit produced no reaction aside from a transient reddening along the lines of scarification.

The reaction observed by low power microscopy in the lungs of the mice injected with variola virus was progressive through the 7th day in respect to the amount of tissue involved as well as the number of animals affected. Pathological changes were observed as early as 24 hours after injection, but only in 4 of the 11 mice examined. 8 of the 11 animals killed on the 4th day, 13 of the 14 killed on the 5th day, and all of the 12 killed on the 7th day showed pulmonary abnormalities.

The normal lung at a magnification of 7 diameters resembles a confined suspension of tiny, closely packed droplets, white or faintly tinged with pink. Injected mice show patchy translucent areas of consolidation which are sharply demarcated from the normal tissue. At first these are small and colorless or grayish, blending perfectly with the involved portions of the lung on visual examination, and commonly found at the attachment of the lobes. Later they increase in size and number, coming to occupy a considerable portion of the lung and giving it a piebald appearance. By the 5th to the 7th day the consolidated areas may be congested and pink in color, and if extensive barely detectable visually. Unmagnified, however, the change in the appearance of the lung is slight and very different from the frank reaction characteristic of vaccinia pneumonia and the native pneumonias of the mouse. As already noted the lungs were sometimes normal in appearance even though virus was present. At the height of the reaction, on the 7th day, virus was no longer recoverable from most of the animals. There was no indication that the lung reaction was referable to the foreign cells or proteins of the egg membrane, the lungs of mice injected with membranes in the absence of virus being normal microscopically.

Histologically the reaction in the lung was characterized chiefly by an interstitial congestion and an infiltration of lymphocytes and mononuclear cells both as cuffs around bronchioles and as solid accumulations or islands, often adjacent to the pleural surface. In general there was little involvement of the alveoli, although in some sections groups of air sacs were filled with erythrocytes or fluid with mononuclear cells. These changes were most noticeable in lungs removed on the 4th through the 7th day. In sections from lungs removed about the 4th day there was sometimes an indication of a proliferation of the bronchial epithelium, solid plugs of necrotic cells being present in the lumen. Polynuclear leucocytes were rarely present at any time and inclusion bodies were never demonstrable.

DISCUSSION

The activity of the virus isolated by us from a human skin eruption diagnosed as smallpox is similar to that of variola strains studied by others. In embryonated eggs it behaves like the virus propagated by Lazarus,

Eddie, and Meyer (4). They commented on the inconstancy of the lesions produced in the membrane by their strain which was carried through 45 egg passages. A similar variability was noted with the present virus up to the 50th passage. The inactivity of the virus on the skin of the rabbit on primary injection is in agreement with the observations of Gordon (6), Horgan (1), and others. There was no indication, however, of a transformation to vaccinia virus on passage through the testis of the rabbit, but that variola virus behaves in this way is still uncertain. The cutaneous eruption which the virus produces in the monkey is essentially like that of the alastrim strains reported by Gordon (6) but apparently less severe than the reaction of his virus from confluent smallpox. The outcome of the protection tests in the monkey agrees with Gordon's (6) finding that recovery from variola affords complete protection against the homologous virus but only partial protection against vaccinia. We believe that these characteristics are sufficient to establish the virus as variola and hence designate it as such.

The present strain of variola virus was also inert in the skin of the mouse and on nasal instillation was much less active than vaccinia virus. Unlike the latter it provoked no symptoms and regularly failed to become established on the nasal mucosa. Beginning with the 64th egg passage it was found that the virus was recoverable from the lung through the 5th day after introduction by the nasal route, and that its residence in the lung coincided with the appearance of well marked pathological changes. These changes were progressive, reaching their height on the 7th day at which time the incidence of recovery of the virus was low.

Whether or not the virus actually multiplied in the lung during its period of residence cannot be said from the present evidence. The results of two titrations indicate that there was no increase in titer beyond that originally introduced. The limiting dilution on subinoculation in embryonated eggs was 10^{-5} , 1 hour after injection, indicative of the amount actually drawn into the lung, and was still 10^{-5} on the 4th day. That the virus did multiply to a limited extent seems a reasonable assumption in view of the stationary titer, particularly since this was maintained under adverse conditions, but there is no actual proof. Whether the maintenance of the virus in the lung is due in part to multiplication or solely to survival, its establishment there is accomplished only when the initial concentration is high.

It is also uncertain whether this short maintenance of the virus in the lung was characteristic of it from the beginning or was a property acquired by adaptation in the egg as the result of long continued passage. Adaptive changes referable to egg cultivation are commonly retrogressive at least in respect to virulence. It is true, however, that the virus did undergo a

change on continued passage as indicated by the more uniform reaction in the membrane after the 50th transfer. Whatever the nature of this change, it was in no sense a transformation. The virus is still basically the same after 85 passages in embryonated eggs and is quite unlike vaccinia virus. There is no indication either that the nature of the virus was altered by its residence in the lung of the mouse. It is planned to continue the egg passages and mouse transfers to determine whether its present identity will be preserved.

SUMMARY

Variola virus was cultivated in embryonated eggs from smallpox crusts and maintained through 85 passages. Therein it produced foci of cellular proliferation and necrosis on the chorioallantoic membrane but did not affect the embryo.

The virus from egg cultures was inactive in the skin of the rabbit on primary injection and in the testis both initially and on passage. In the monkey it provoked a cutaneous eruption of short duration after an incubation period of 5 days.

On nasal instillation in the mouse the virus caused no symptoms and failed to survive on the mucous membrane of the upper air passages. Beginning with the 64th egg passage it was regularly recoverable from the lung, on subinoculation in eggs, through the 5th day and occasionally through the 7th day. Its presence in the lung was attended by progressive pathological changes.

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EXPLANATION OF PLATES

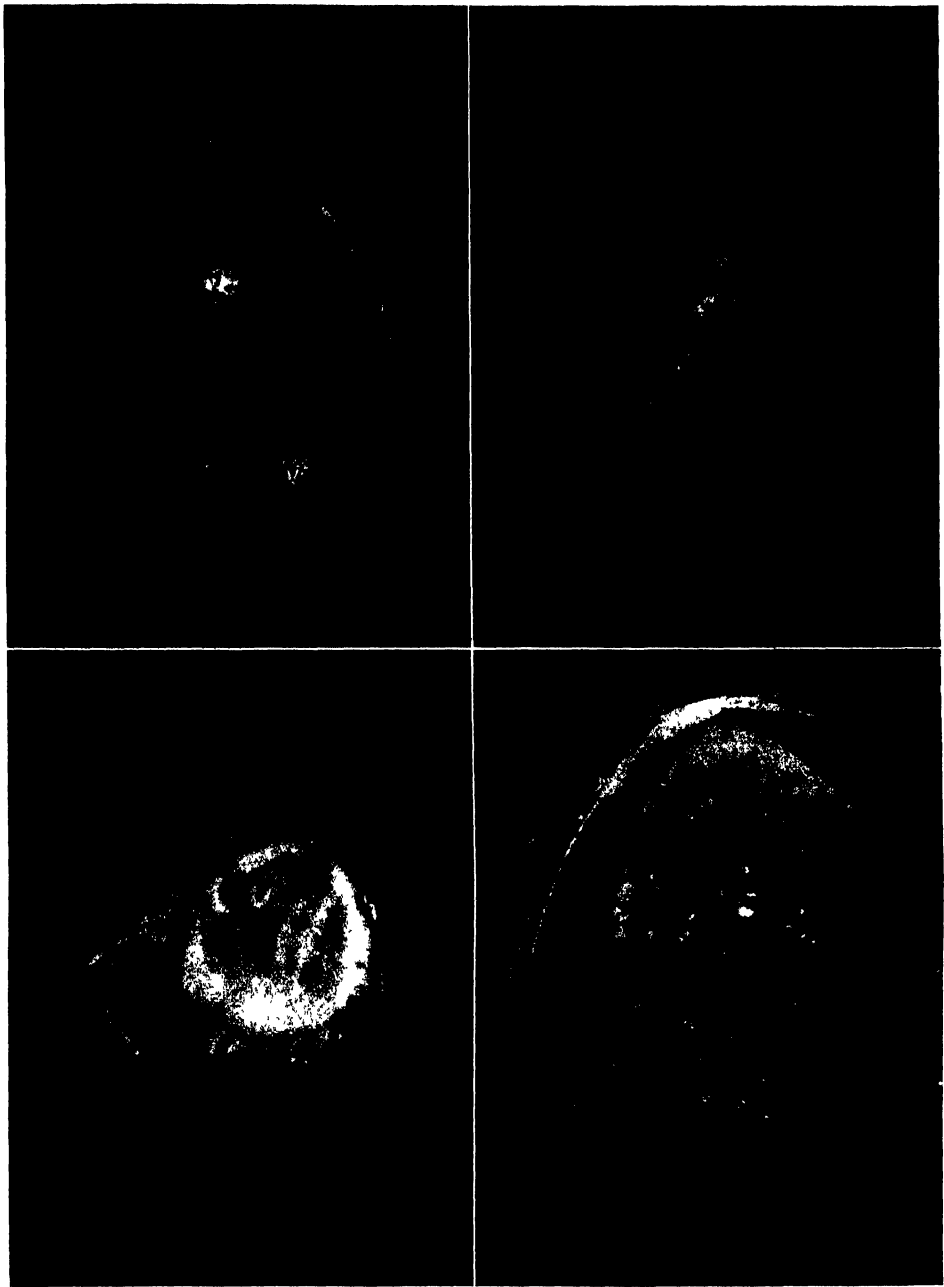
PLATE 8

FIG. 1. Small cluster of foci in membrane from egg inoculated with variola virus, type of reaction often encountered in early passages.

FIG. 2. Discrete foci in membrane removed on the 3rd day after inoculation.

FIG. 3. Coalesced foci in membrane removed on the 4th day after inoculation.

FIG. 4. Streaky foci in membrane from an egg inoculated with a 4-day lung suspension. All of these membranes are approximately normal size.



Photographed by J. A. Carlile

(Nelson: Pox viruses in respiratory tract. II)

PLATE 9

FIG. 5. Elementary bodies in membrane removed on the 3rd day after inoculation. Morosow preparation. $\times 2100$.

FIG. 6. Left lobe of lung removed on the 5th day from a mouse inoculated with variola virus. $\times 6$.

FIG. 7. Section of lung removed on the 7th day from an inoculated mouse. Phloxin-methylene blue stain $\times 120$.



Photographed by J. A. Carlile

(Nelson: Pox viruses in respiratory tract. II)

FAMILIAL MAMMARY TUMORS IN THE RABBIT

I. CLINICAL HISTORY

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PLATES 10 TO 12

(Received for publication, May 10, 1939)

Spontaneous tumors of the breast are known to occur with high frequency in certain species of animals, notably the mouse and the dog but, on the other hand, very few cases have been reported in the rabbit. A report from this laboratory in 1938 cited 10 cases (1) but aside from this series only 3 instances could be found in a review of the literature (2-4). These were accidental findings and there were no clinical notes or antemortem pathological studies to correlate autopsy findings with factors which might have thrown light on the origin of the tumors.

The study of breast tumors in the rabbit to be reported in this and succeeding papers represents a development of planned experiments which constitute one phase of a more comprehensive investigation of constitutional problems based on an animal population of known composition. It is necessary to refer briefly to the origin and development of this work so that the study of the tumors may be viewed in proper relation to other experiments.

General Plan

This series of investigations had its inception in 1929 with the organization of a breeding colony for the study of constitutional problems (5). At that time no known material was available for the study of spontaneous neoplastic diseases in the rabbit and it was hoped that among the animals comprising the population suitable material could be developed. Animals of presumptive tumor age were given a place in the colony organization and routine examinations were made for the purpose of detecting neoplastic conditions. The study of breast tumors and of other neoplastic diseases began, therefore, as a population survey.

The first breast abnormality was found in the fall of 1930 in a Belgian hare purchased as foundation stock. This was a cystic condition comparable with that to be described in connection with Fig. 4, but complicated by an extensive suppurative process, which eventually necessitated the disposal of the animal. A pure bred son and daughter

together with their sire and 2 other daughters from an outcross mating constituted the nucleus from which the present tumor stocks were developed.

The inherent tumor potentialities of these animals were unknown but the recognition of other functional disorders had marked this family of Belgian hares as the most important group in the colony. It was also the most difficult to maintain. Every effort was made to perpetuate the line in pure form but this was impossible and from time to time other strains of Belgians were introduced and crossed with the original line. Outcrosses with other racial groups were also made in connection with the study of the functional disorders mentioned above. The family of animals from which the study of breast tumors was eventually developed thus consisted of a small group of pure Belgians and a much larger group of hybrids composed chiefly of primary crosses between English and Belgian stocks and animals derived from further crosses of these hybrids among themselves and with the parent Belgian line.

English rabbits were selected for the crosses because they represented the nearest approach to normal of any racial group in the population and a type nearest that of the Belgian. It was subsequently discovered, however, that in this way one breast tumor line was crossed with another. The English population was later found to be a second source of breast tumors but, as will be pointed out, tumors of the two lines differ in type and in the age of development.

No mammary tumors were detected until 1934. Small groups of animals from numerous racial and family groups had been held for observation over periods of several years as parts of the working population. On several occasions, heavy losses occurred from outbreaks of epidemic disease and in all cases the Belgian population suffered most so that potential tumor animals were continually lost before reaching tumor age.

In the spring of 1934, the first abnormality of the present series, which consisted of a cystic breast condition, was discovered in the course of a routine population check. The animal was used at once for the breeding of F_1 hybrids, while its nearest relatives were preserved to provide material for tumor studies if the subsequent course of events in this animal should warrant such an undertaking.

In 1935, the colony was moved from New York to Princeton and in a short time after other animals developed mammary tumors. These occurred not only in the Belgian population but also in the family of English rabbits referred to above and in hybrid crosses. Meanwhile, the condition in the first animal progressed to cancer.

With the material thus provided, plans were made for a more direct and comprehensive investigation of the genesis of spontaneous neoplasia. Genetic studies¹ and transplantation experiments were included in this program. Tumor animals were used for F_1 and backcross matings. On the basis of evidence obtained from an analysis of pedigrees, matings among other animals of this group were made with a view to increasing the number of probable tumor animals. For a while, the English as well as the Belgian line was developed; but both lines could not be accommodated and eventually work with the English stocks was discontinued as available evidence indicated that these animals would have to be held from 1 to 2 years longer than the Belgians before end results could be obtained.

¹ Genetic experiments are being conducted by Dr. Pearce and consideration of the influence of hereditary factors in these papers will be limited to a pedigree analysis.

It has been necessary to pursue these studies as experiments coordinated with other problems. Still, it was believed that the study of neoplastic diseases in relation to other functional disorders in the general population possessed some advantages. Tumors of the breast have been investigated, therefore, as a population study with especial reference to the bearing of inherent constitutional peculiarities on the genesis of mammary tumors. It so happens that the family group concerned presents the greatest variety of functional disorders of any group in the entire population and any effort to determine whether these associated conditions possessed significance would require a control such as that supplied by the remainder of the population. Moreover, it seemed that this method of procedure placed the problem on a footing more nearly comparable with conditions surrounding the development of cancer in human populations, with the added advantage of a more intimate knowledge of members of the experimental population and the opportunity to employ measures of control as circumstances demanded.

In this connection, it should be pointed out that what has been said with reference to the association of breast tumors and functional disorders is equally applicable to other neoplastic diseases which have been encountered in this laboratory. In practically all cases, interest in some striking or profound functional disorder has prepared the way for the discovery of the neoplastic condition. In other words, tumors have developed largely in that portion of the population which is classed as abnormal rather than among the so called normal groups. This may be merely coincidence but it was noted in the case of uterine tumors and its significance will be considered in connection with breast tumors.

During the past few years a comparatively large number of spontaneous tumors of the breast has been obtained. The tumors have been investigated systematically through their entire course of development, including a clinical study of breast changes, the gross and microscopic pathology of the conditions as revealed by biopsy at frequent intervals and complete autopsies on those animals which have died or have been killed. In addition, an effort has been made to correlate the results thus obtained with the functional behavior of the host.

The present paper will be limited to the clinical aspects of spontaneous tumors. This will be followed by a second paper dealing with gross and microscopic pathology, while a third paper will be concerned with coincident changes in other organs and with other factors which may have influenced the occurrence and course of the spontaneous growths. The successful transplantation of the tumors and their growth characteristics in animals of the same and of foreign species will be described at a later date.

Materials and Methods

The organization and management of the colony has been described elsewhere and need not be repeated here (6). It should be noted, however, that the management of the tumor-producing part of the population differed from that of the colony as a whole in only two respects, namely, the use of extraordinary precautions to safeguard tumor animals and the feeding of green food to this group at more frequent intervals, particularly during the past year.

The material for this study was derived from several classes of experiments. As already stated, long series of breeding experiments were carried out in an effort to concentrate or fix inherent constitutional characters in numerous pure bred lines of rabbits. When a source of tumor material had been found in this way, a second series of breeding experiments was undertaken to preserve this material and to provide a more abundant supply of tumors.

Another class of experiments was directed toward the correlation of clinical and pathological alterations in breast tissues from the earliest recognizable changes to the development of neoplasia and eventual malignancy. Such experiments involved systematic examinations of large numbers of animals with the performance of biopsies at frequent intervals on those found to have developed mammary abnormalities. Biopsies were done under ether anesthesia. In conjunction with these experiments, attempts were made to transplant selected tumors at various stages in their development as a biological criterion of self sufficiency or capacity for independent growth. Experiments of a converse nature were also undertaken to determine the response of tumor-bearing animals to the transplantation of their own or of another tumor capable of growing in normal animals.

A third class of experiments was carried out in an effort to determine whether any relation existed between the occurrence and subsequent course of mammary tumors on the one hand and inherent functional disorders or disturbances of normal function on the other. These experiments have followed two directions: first, a comparison of tumor-bearing lines and tumor-bearing animals with non-tumor-bearing stocks on a genetic basis, and second, a comparison of the functional behavior of tumor animals of a given group with animals which did not develop tumors. Comparisons of the second type have been concerned chiefly with reproductive phenomena. In addition, the influence of reproductive cycles and the relation of disturbances in these functions to the occurrence of mammary disorders, the relation of mammary disorders to the occurrence of neoplasia and the evolution of malignancy have been investigated.

Thus far 29 animals presenting mammary abnormalities known to be connected with the development of neoplastic changes have been studied for various periods of time. 11 of these died of intercurrent disease or were killed before the ultimate outcome of the condition could be determined. 7 additional animals are still under observation, while in a third group of 11 animals the breast changes were followed from an early stage to cancer; 10 of these animals have come to autopsy and 1 is still living.

This group of animals does not represent the ultimate incidence of breast tumors in the population or the probable end result of the breast condition in all cases. A preliminary period of observation was fixed at 3 years, but many animals were discarded before the expiration of this period because they could not be used for breeding purposes. Others were discarded at the age of 3 years if no abnormality had developed and they could not be used advantageously for other purposes. The time limit was based on the behavior of the Belgian population. It is certain, therefore, that some animals were eliminated which would have developed tumors if they could have been held for further observation. In like manner, some animals with breast tumors died from intercurrent disease; others became debilitated or developed conditions which overshadowed the tumor growth and were killed when it appeared that no further information could be obtained or that the animal might die at a time when an autopsy could not be performed.

In the first group of cases studied, clinical examination was supplemented by biopsy

at such times as clinical changes in the breast seemed to warrant, but the study of material obtained in this manner showed that a considerable period of time separated the occurrence of microscopic structural alterations and the appearance of indicative clinical signs. The material was not sufficient to fix the exact time limits of the various histological stages of the disorder and an attempt was made in recent cases to determine more precise time relations by performing biopsies at more frequent intervals. Biopsies were performed in the periphery of the breast in order to minimize damage to the main ducts and to avoid histological alterations which might result from interference with proper drainage during lactation.

Complete autopsies were performed in all cases and all organs were sectioned and studied microscopically. Routine biopsy and autopsy sections were fixed in Petrunkevitch's solution and pituitary glands were fixed in Susa's solution. General sections were stained with hematoxylin and eosin and pituitary glands with a modification of Mallory's aniline blue method. Contiguous breast sections were stained with hematoxylin and eosin and with Weigert's elastic tissue stain.

Clinical Classification of Tumors

Two distinct morphological types of tumor which differ in mode of development and biological characteristics have been observed in the breasts of rabbits. A distinctive antecedent mammary history further differentiates one type and is used as a basis of classification in the presentation of data in Tables I and II. Table I records cases with complete, as well as incomplete, clinical courses and, in the latter class, a number of animals without neoplasia but with early breast changes are included. On the other hand, all animals bearing tumors of the type listed in the second table have died and no new instances have been discovered.

The development of neoplasia was preceded by a history of cystic disease in 19 cases, while in 4 cases no antecedent mammary changes were observed. In 2 instances the presence of tumor was first noted at autopsy and while the course of the disorder was not followed during life, the coexistence of advanced cystic disease suggested a developmental history similar to that of the first group of cases.

Tumors Originating at the Site of Preexisting Cystic Disease

The course of the mammary disorder leading to neoplasia was under clinical observation from the inception of cystic changes in the majority of cases. The sequence of events throughout the period of study was similar in all cases and, in instances in which death occurred before invasion, the similarity of the observed developmental course suggested that with continued life these growths would also have become cancerous. In order to avoid repetition, therefore, individual case histories will not be cited in the present section but will be dealt with collectively and the disorder will be

TABLE I
Tumors Originating at the Site of Preexisting Cystic Disease

No.	Age at inception of disorder	Nature of first abnormality	Period of observation	Eventual fate	Condition at death	Terminal or present status
	mos.		mos.			
B16-2	29	Cystic disease	25	Killed	Moribund	Adenocarcinoma with metastasis
B178	25	" "	19	"	Failing	Adenocarcinoma
B234-3	30	" "	7	"	Moribund	Medullary carcinoma with metastasis
B240-2	29	" "	16	Died		Adenocarcinoma with metastasis
B346-3	27	" "	13	Killed	Moribund	Anastomosing papillomata
B348-1 (BE)	20	" "	7	"	Good	Adenocarcinoma
BE28-3	36	" "	6	"	Moribund	Anastomosing papillomata
BE44-2	29	" "	2	Died		Adenocarcinoma
X1966-4	38	" "	9	"		Sessile epithelial neoplasia
X5074-2	27	" "	7	Killed	Moribund	Anastomosing papillomata
X6106-3	17	" "	21	Died		Adenocarcinoma
X9755-3	10	" "	11	"		Anastomosing papillomata
X2594-3	36	" "	7	Killed	Good	" "
X1984-4	43	" "	8	"	"	Uniradicular papillomata
X1539-1	35	" "	17	"	Poor	Anastomosing papillomata
B249-3	35	*		"	"	Small solitary anastomosing papilloma
B306-3	23	*		"	"	Sessile epithelial neoplasia
EB2-2	29	Cystic disease	29	Living		Adenocarcinoma
BE75-6	43	" "	4	"		Sessile epithelial neoplasia
X5943-9	44	" "	3	"		Anastomosing papillomata
X7634-6	33	" "	3	"		Cystic disease
X7768-3	30	" "	6	"		" "
X8157-1	19	" "	16	"		Anastomosing papilloma
X10169-1	11	" "	15	"		Cystic disease
X10557-3	10	" "	16	"		" "

* Breast abnormality in which cystic changes predominated, first discovered at autopsy.

TABLE II
Tumors Not Associated with Preexisting Cystic Disease

No.	Age at inception of disorder	Nature of first abnormality	Period of observation	Eventual fate	Condition at death	Terminal or present status
	mos.		mos.			
B57-2	47	Tumor	3	Killed	Good	Adenoma
E33-5	51	"	16	Died		Adenocarcinoma with metastasis
E126-2	34	"	12	Killed	Poor	Adenocarcinoma
T36-1	42	"	16	"	Moribund	Adenocarcinoma with metastasis

described as it occurred in typical progressive instances beginning with cystic disease and terminating with metastasis.

The sequence of events was also followed by biopsy and examination of the tissue shows the advance of the disease in three more or less distinct stages which offer a convenient division of the clinical course for descriptive purposes. It should be emphasized, however, that, while the different stages apparently form parts of a continuous disease process, great variation occurs in the rapidity of progress from one phase to another and under certain conditions the disorder may be arrested for long periods of time in any one of its developmental stages. The factors influencing this variation have been the subject of special study and will be discussed in a later section together with other pertinent findings derived from an investigation of individual cases.

Cystic Disease.—The first indication of breast abnormality may occur either as a sudden and intense engorgement involving the entire mammary system (Fig. 1) or as a slight granular enlargement in a segment of a single gland. Such engorgement, so far as known, is associated with a recent infertile mating, or an estrous period, while the primary granular changes are found in resting animals. The failure to note preliminary engorgement in the latter cases, however, does not preclude its possible occurrence in a masked form during a preceding period of pregnancy hypertrophy or lactation. In any case, the ensuing sequence of events proceeds in a similar manner.

Engorgement may persist and increase in severity for 2 or more weeks (Fig. 2). The breast tissue appears white through the stretched skin but, toward the end of the period the distended glands become translucent, and clear, colorless fluid instead of milk may be expressed from the nipples. Biopsy shows the presence of large, dilated spaces filled with thin white or colorless fluid. The mammary tissue is delicate, easily torn and not fibrous. Many of the dilated spaces intercommunicate and operation usually results in a temporary collapse of the breast with recurrent distension following in a day or two.

The engorgement subsides suddenly in the majority of cases and resolution may take place within the course of a few days. There may be a complete return to a normal condition but occasionally the breasts remain enlarged (Fig. 3). In such cases, the glands are soft and doughy to palpation and operation shows an increase in parenchyma with a small amount of fluid secretion. Usually extremely small granular thickenings may be found on careful examination in the periphery of one or more breasts following subsidence of the primary distension.

The engorgement eventually reappears after a variable period during which the animal may or may not have been remated. In the majority of cases, the above mentioned relationship to mating obtains and if pregnancy results, pregnancy hypertrophy and lactation follow the primary engorgement and the resulting litter nurses and appears to thrive. In instances in which the engorgement recurs during a resting phase, its appearance has been observed to coincide with manifestations of heat and its resolution to follow a fertile mating. In still other cases the subsidence of engorgement has been found to occur simultaneously with a deterioration in the general health of the animal and its reappearance to follow a gain in weight and a return to better physical condition.

The cycle of engorgement and return to normal may be repeated a number of times before residuary changes are noted, but eventually minute shotty nodules persist. The nodules may be scattered in small groups throughout the mammary tissue with no constant relation to the architecture of the breast and may remain as isolated lesions throughout the early course of the disorder. In other regions the nodules are aggregated in firm, granular areas which occupy a small, peripheral mammary segment and, with progress of the disease, gradually extend to form a flattened pancake-like mass with distinct borders which encompass the greater part of the gland (Fig. 4). It should be emphasized that the granular changes are not localized but occur throughout the mammary tissue. All regions are not equally affected and, while the changes in some breasts produce masses apparent to the naked eye, their presence in other glands can only be determined by careful palpation.

With enlargement of the nodules their cystic nature becomes apparent: many appear blue but others show no color through the shaved, overlying skin. Fluctuation can be elicited and, occasionally, rupture with a discharge of clear fluid from the nipple results from excessive pressure applied during palpation. Individual cysts may attain a diameter of 2 cm. or more, but smaller cysts ranging to a pinhead in size make up the bulk of the larger breast masses. The nipples are occasionally affected by the cystic change and may be greatly distended with the skin stretched to paper thinness. Rupture and collapse with a spurt of colorless fluid follows minor trauma in such cases.

At biopsy, individual cysts are intimately attached to surrounding tissues and cannot be shelled out. The blue cysts are thin walled and easily ruptured, while other cysts are thick walled and resist considerable pressure. The large pancake-like masses of granular tissue are easily distinguished from normal breast. They appear greyish brown in color with small lighter and darker focal areas and are well demarcated from the surrounding tissues by clear cut, rounded borders. Their substance is made up of numerous small, cystic nodules which on section may contain clear, colorless fluid or dark brown, inspissated material.

The clinical picture does not remain constant but is characterized by periods of progression and regression. Engorgement may recur at any time and obscure the cystic changes. It may be generalized or localized to a single region and its subsidence is associated with the appearance of new cysts and an increase in the size of existing nodules. Large dilated areas filled with milk persist for considerable periods of time and the secretion becomes thick and semisolid. Periods of regression during which existing cysts decrease in size and may entirely disappear occur without any observable change in the condition of the animal. In other instances temporary regression is associated with a decline in physical condition and is succeeded by recurrence following recovery.

The stage of cystic disease is of extremely irregular duration but eventually, biopsy examination shows the presence of neoplasia in widely scattered areas.

Stage of Non-Invasive Neoplasia.—It is not possible to detect beginning neoplasia clinically. The process occurs with equal frequency in microscopic and in large cysts and the size of individual nodules or the extent of breast involvement cannot be used as criteria in this respect.

In advanced cases, nodules which were previously soft and cystic become firm and non-fluctuant and occasionally bloody fluid can be expressed from a nipple. The masses in individual breasts increase at an unequal rate but, at this stage, the involvement of

all mammary tissue is obvious to casual examination (Fig. 5). The enlargement of some breasts is no greater than is observed in normal lactation but others become pendulous and attain such a size that they are not raised from the cage floor when the animal is erect and, as a consequence of continuous trauma incident to normal activity, may become infected.

At biopsy, the breast tissue is extremely irregular in appearance. Cysts of all dimensions ranging from 3 cm. in diameter to the size of a pinhead are found in close proximity. Some are completely filled with greyish, fleshy tissue, others show mural excrescences of the same description and still others contain only clear fluid or thick, brown, inspissated material. The intercystic tissue is firm and fibrous.

Engorgement may recur during this stage but is usually localized in one or more breasts. Resolution is followed by the appearance of a fresh crop of cystic nodules and all stages of the disorder from minute, granular thickenings to large, firm, pendulous masses may be found in a single animal. The degree of visual breast involvement and the size of individual masses vary widely. Large nodules which on histological examination have shown neoplastic growth may be reduced to a small fraction of their previous mass in the course of a few weeks and, on the other hand, may enlarge with equal rapidity. A particular mammary region may show the most pronounced lesions at one period, but at a subsequent examination less than 1 month later, the site of predominant involvement may be shifted to a different area.

After a variable period of time during which the condition in one or more breasts becomes stabilized, microscopic examination shows areas of active invasion. This occurrence may not be reflected in indicative physical changes for a considerable period of time but eventually signs of invasive growth dominate the clinical picture.

Stage of Invasive Neoplasia.—Retraction of the nipple, fixation to the skin and muscle and an increased growth rate are the characteristic clinical features of this stage (Figs. 6 and 7). Small areas of softening may occur and ulceration of the overlying skin frequently follows. Such alterations usually appear in the breast in which antecedent physical changes were most pronounced and may be entirely limited to that breast, but occasionally other glands in remote, as well as adjacent, positions show signs of invasive growth.

The main tumor mass increases at an uneven rate with periods of reduced and accelerated growth which correspond with the general physical condition of the animal and to a lesser but detectable degree with the season of year. The mass may present a firm, nodular surface with areas of board-like hardness or rounded contours with a soft, doughy consistency depending on the histological type of growth. It is a characteristic feature of the condition that the growth of nodules in other breasts is slow and abortive in the presence of a rapidly increasing tumor mass. In the final stages, therefore, the typical picture is that of a single, large, rapidly growing mass with smaller, less active nodules scattered throughout the remainder of the mammary system (Fig. 8). Metastatic areas of growth can often be palpated in the regional lymph nodes.

At biopsy, the main mass may be fleshy or fibrous and present areas of cyst formation or of necrosis. Nodules in other breasts may be purely cystic or filled with fleshy tissue limited to the cyst wall or extending diffusely into the surrounding region.

The animals exhibit no sign of cachexia other than a progressive loss of weight and generally remain alert and active until shortly before death. The duration of the dis-

order is extremely variable. The entire clinical course from the beginning of cystic changes to death with metastasis was completed in 7 months in one instance and was prolonged to 25 months in another.

Tumors Not Associated with Preexisting Cystic Disease

Tumors of this class are less common and at the present time only four have come under observation. The differentiating developmental feature of these growths is the absence of preexisting cystic disease, but it should be emphasized that during their course cystic changes frequently occur in structures formed by the tumor elements and are occasionally found in the neighboring mammary tissue. These changes remain inconspicuous, however, and their detection depends on microscopic examination.

In their earliest stages the tumors appeared as solitary, pea-sized, elastic nodules freely movable in normal breast tissue. In all but one instance the neoplastic process remained confined to a single nodule and other breasts were not involved except by extension from the primary growth. This case, however, was characterized by the occurrence of numerous, smaller, rounded masses scattered throughout the mammary tissue and resembling the main growth in all physical characteristics.

One animal was killed after 10 weeks of observation when the nodule had reached a diameter of approximately 2 cm. The remaining animals were held from 12 to 16 months but in no instance did the tumors attain a size comparable with those of the previous series. Growth was slow but progressive and the periods of acceleration and retardation which characterized the preceding tumors were not apparent. The nodules remained firm, rounded and elastic and attachment to surrounding structures did not occur until a late stage.

In one instance a metastatic nodule appeared in a breast of the opposite side, 10 months after discovery of the primary tumor (Fig. 9) and in this case and in one other, visceral metastases were found at death after a clinical course of 16 months.

SUMMARY

The clinical histories of two different types of familial mammary cancer in the rabbit have been described. In one type, the first clinical sign of breast abnormality was a sudden and intense engorgement and thereafter the disorder passed through stages of cyst formation and benign neoplasia to cancer with metastasis. In the second type, neoplasia originated in clinically normal breast tissue and there was no history of antecedent mammary abnormality.

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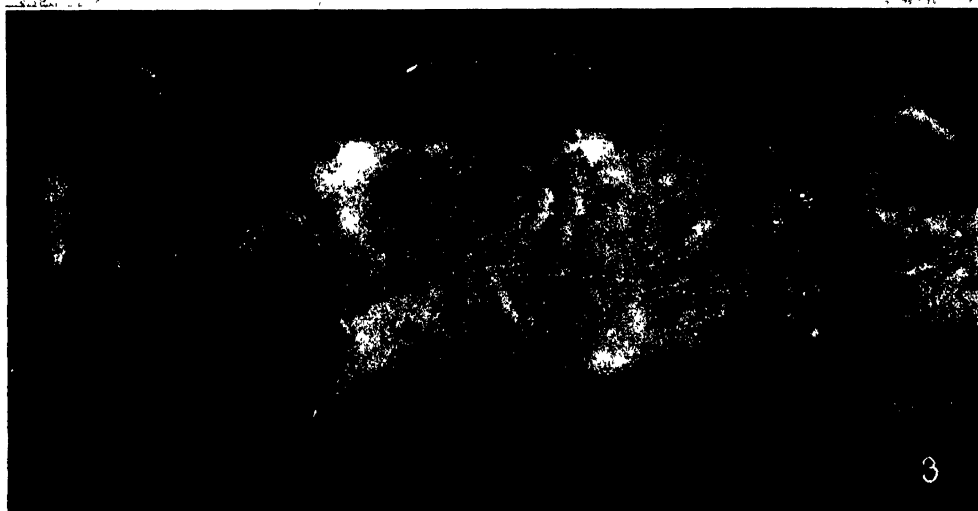
EXPLANATION OF PLATES

PLATE 10

FIG. 1. X10169-1. Primary engorgement involving the entire mammary system. The trauma in the third right breast is a result of a recent biopsy examination. $\times 0.4$.

FIG. 2. X6106-3. Persistent, increasing engorgement.

FIG. 3. X6106-3. Photograph taken 1 month later. The acute engorgement has subsided but all breasts show residual enlargement. Beginning granular changes are present in the peripheral region of many glands and the nipple of the fourth left breast shows typical cystic dilatation. $\times 0.4$.



Photographed by J. A. Carlile

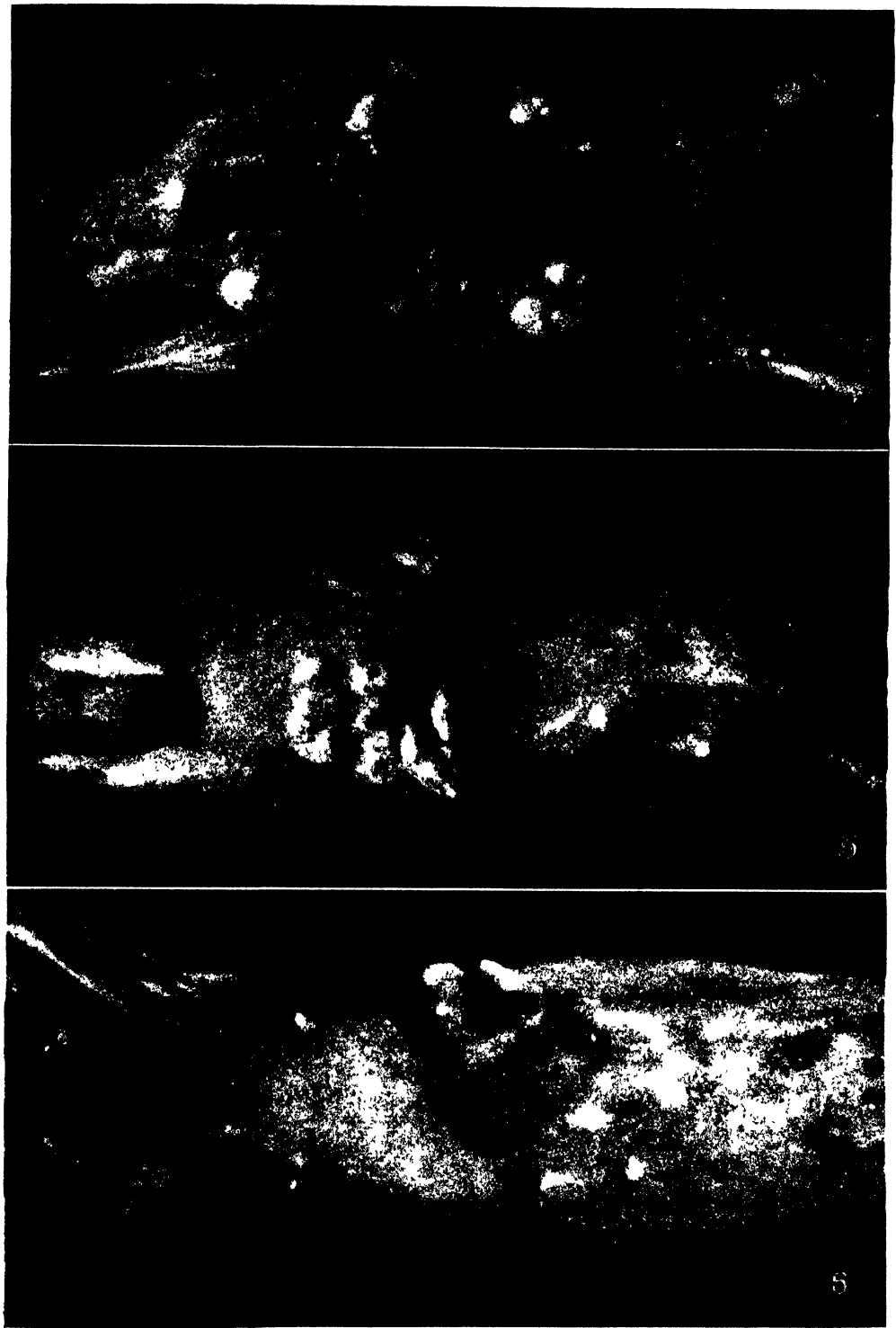
(Greene: Familial mammary tumors in rabbit. 1)

PLATE 11

FIG. 4. X8157-1. Photograph taken 4 months after inception of the disorder. Many breasts show residual enlargement and contain isolated cystic nodules. The fourth right breast contains a mass of characteristic hypertrophic, granular tissue. The first breasts of the two sides are not shown in the photograph. There is a supernumerary breast on the right side. $\times 0.4$.

FIG. 5. B178. Photograph taken 6 months after inception of the disorder. The tumors in the third breasts of both sides are firm, non-fluctuant and freely movable. Biopsy examination demonstrated the presence of non-invasive neoplasia. Other breasts contain smaller neoplastic nodules, cysts and localized areas of engorgement. $\times 0.35$.

FIG. 6. B240. Photograph taken 7 months after inception of the disorder. The tumor in the third left breast has invaded the skin and appears externally in two areas. Despite local invasion the animal remained in good physical condition and death with metastasis did not occur until after the lapse of a year. The changes in other breasts had almost completely regressed but subsequently reappeared. $\times 0.55$.



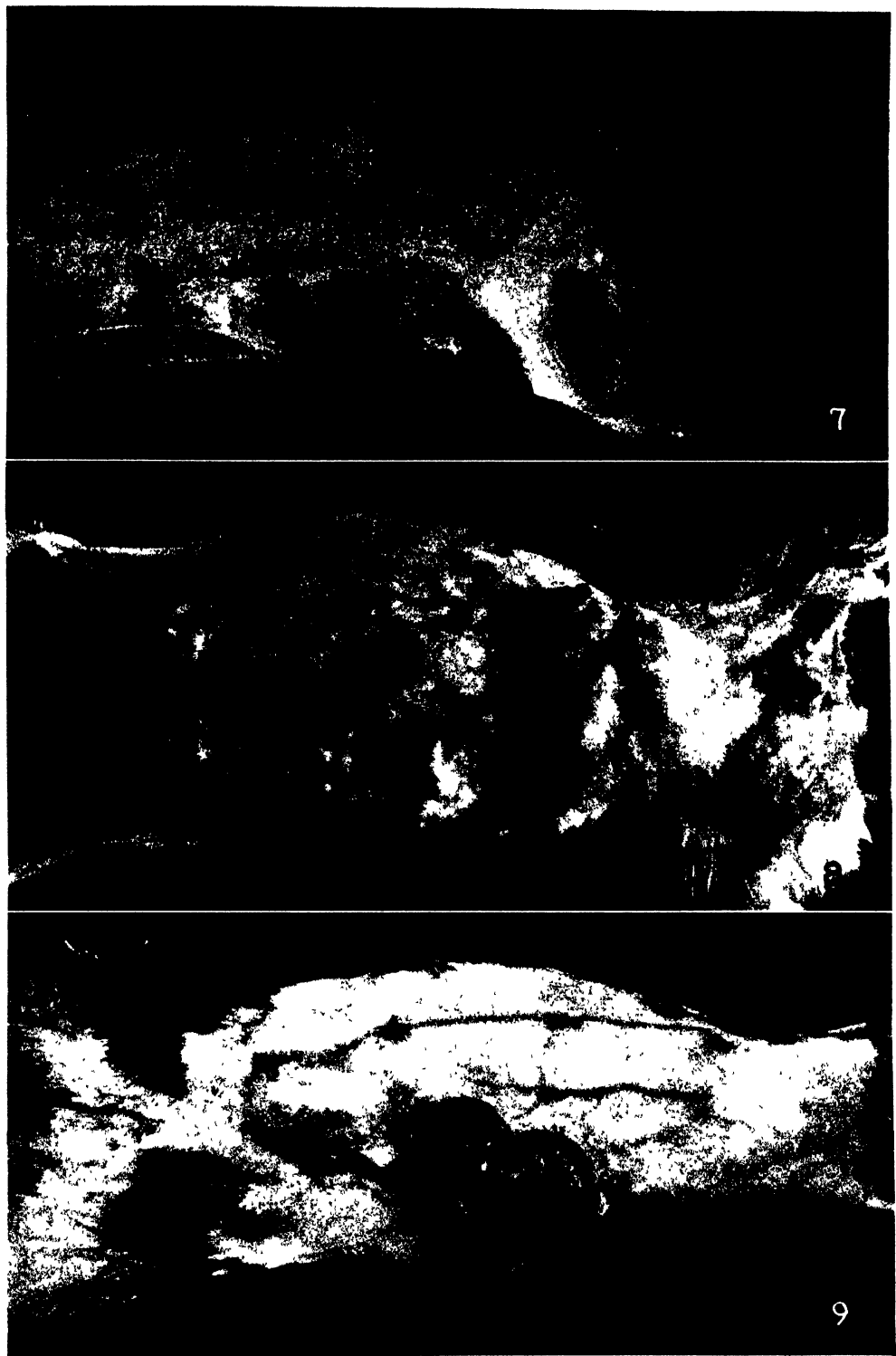
Photographed by J. A. Carlile

PLATE 12

FIG. 7. B234-3. Photograph taken 7 months after inception of the disorder. The mass in the first left breast is soft and doughy in consistency. Microscopic examination showed the presence of a medullary carcinoma. Death with metastasis occurred 2 weeks after this picture was taken. $\times 0.55$.

FIG. 8. EB2-2. Photograph taken 26 months after inception of the disorder. All stages of the disorder are present in different regions. Directly below the second right breast is an area of engorgement. Nodules, scattered throughout the mammary system, are purely cystic in character or contain multiradicular papillomata. The first left breast is the seat of an invasive growth. The rabbit is living at the present time and the mammary disorder has been present for 29 months, exactly half the animal's life. $\times 0.4$.

FIG. 9. T36-1. Photograph taken 16 months after discovery of the primary tumor nodule. The primary tumor is the lower mass shown in the picture, while the upper mass is a secondary growth of 6 months duration. A direct lymphatic extension from the primary to the secondary tumor was traced at autopsy. The primary growth shows no clinical evidence of malignancy but the secondary tumor has invaded the skin and is attached to the underlying muscle. Other breasts are normal in appearance. Metastases are present in the lymph nodes of the left axilla and groin. $\times 0.4$.



Photographed by J. A. Carlile

(Greene: Familial mammary tumors in rabbit. I)

FAMILIAL MAMMARY TUMORS IN THE RABBIT

II. GROSS AND MICROSCOPIC PATHOLOGY

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PLATES 13 TO 17

(Received for publication, May 10, 1939)

The clinical histories of two different types of mammary cancer in the rabbit were described in the preceding paper. It was observed that the differentiating clinical feature of the tumors was the occurrence of a characteristic antecedent breast history in one type. Pathological study supports this distinction and discloses additional differences in morphology and mode of development. Repeated examination of mammary tissue obtained during different developmental stages of the disorder shows the presence of a continued disease process which in one case is neoplastic from the start, but in the other originates as an apparently unrelated pathological state and progresses through non-invasive neoplasia to cancer. The object of the present paper is to describe the general histological changes which characterize the various developmental stages.

Histological Classification of Tumors

The relation of human and animal tumors is not entirely clear and the applicability of the histological criteria used in human pathology to a differentiation of benign and malignant growths in the rabbit is open to question. The use of such criteria is based entirely on experience which does not necessarily carry over to other species. Since experience with rabbit tumors is limited and the significance of abnormal cell structure to the eventual fate of the growth and of the animal has not been determined with certainty, it seems wiser for the present, at least, to adopt a classification based on tissue relations rather than on cell morphology. In the present classification, therefore, growths which may show all the abnormal cellular morphology associated with malignant tumors in man but remain confined within normal boundaries will not be referred to as cancers; this term will be applied only if the neoplastic cells have invaded and appear to have proliferated in foreign locations.

The clinical classification of the tumors based on antecedent breast history is represented on histological examination by distinctive differences in morphology and mode of development. Tumors in cystic breasts arise as papillomata from the epithelial lining of dilated acinar and duct walls and after continued growth form multiple radicles which anastomose with the production of numerous acinus-like structures. Growths of this type are classified as anastomosing papillomata rather than as cyst adenomata to distinguish them from tumors of the second type which originate in normal breasts, appear to arise from a proliferation of true acini, and are more accurately classified as adenomata. 8 of the anastomosing papillomata progressed to cancer, 7 did not show invasive growth at the time of death and 2 are still under observation. The course of the disorder was terminated in 2 instances while the breasts were in a state of sessile epithelial neoplasia, and the condition has progressed to a similar stage in one living animal. In one additional instance, an animal was killed while the papillomata were in an early uniradicular stage. In the second class, 3 adenomata advanced to carcinomata and one showed no invasive changes at autopsy.

Tumors Originating at the Site of Preexisting Cystic Disease

The progress of mammary changes leading to cancer was followed by frequent biopsy examination which showed the occurrence of cystic disease, benign neoplasia and invasion as succeeding events in the breast. In other instances the terminal phase was one of benign neoplasia but the developmental course was identical with that noted in growths which subsequently progressed to cancer. For convenience, therefore, the disorder will be described as it occurred in cases which advanced to invasion and terminated in metastasis.

Cystic Disease.—The stage of engorgement which precedes the occurrence of cystic disease in the majority of cases is characterized microscopically by a marked increase in the number of active gland fields which, however, remain discrete and do not coalesce as in lactation hypertrophy. The interlobular connective tissue is abundant, loose and edematous. Sections obtained from the early granular tissue which persists after the subsidence of engorgement show a condensation of more mature, fibrous connective tissue about gland fields which appear shrunken and smaller. Individual acini are not increased in size, secretion is absent and the epithelium is low and atrophic so that their lumina appear enlarged. At a later period, however, acini in different portions of a gland field are dilated and may contain a pale eosin-staining secretion. The epithelium remains low and atrophic in the great majority of dilated acini, but in scattered focal groups it becomes columnar in shape and deeply basophilic. In such cases, the lumen of the acinus frequently contains desquamated cells (Fig. 1).

With progress of the disease and extension of the focal granular areas into large, pan-cake-like masses, microscopic examination shows an abundance of cysts of varying size and appearance, dilated ducts and normal appearing gland fields. The cysts obviously arise from dilated acini but the mechanics of the dilatation is obscure. Frequently, the duct draining a gland field is widely dilated, yet its acini remain unaffected. All of the acini of a gland field are rarely involved, some are widely dilated but others in an adjacent position are unaltered in structure (Fig. 2). The lining epithelium is low and appears stretched in the majority of cysts, but in other positions in the same or in different fields it is columnar and occasionally more than one cell in depth.

Sections from more advanced cases show pronounced dilatation of the majority of acini in a gland field (Fig. 3). Septal walls are thin and are frequently ruptured so that a whole field may be converted into a single, large cyst. Dilatation is less marked in acini lined by low atrophic epithelium and gland fields made up of such elements do not expand and coalesce but remain discrete and are surrounded by fibrous connective tissue containing numerous elastic fibers. On the other hand, gland fields containing cysts lined by higher epithelium are distorted by expansion and neighboring fields coalesce. The unaffected acini are compressed, hardly recognizable, and may appear as unrelated structures in the connective tissue immediately surrounding the dilated areas. The epithelial lining cells are frequently arranged in multiple layers but remain orderly and show no changes suggestive of neoplasia. The histological features which distinguish ducts and their acini in normal breast tissue are almost entirely lost; both dilated ducts and cystic acini are lined by similar epithelium and the distinctive subepithelial elastic layer can only be detected as scattered fine fibrils in occasional dilated ducts.

Stage of Non-Invasive Neoplasia.—After a variable period of time, early neoplastic foci are found widespread throughout the tissue in both dilated ducts and cystic acini. They may occur as small, sessile growths composed entirely of epithelium or as uniradicular papillomata with a thin, delicate connective tissue stalk (Figs. 4 and 5). They may be found in all breasts and all stages of development are frequently seen in different sections of the same gland.

Purely epithelial growths are not found in later stages and it is assumed that they excite a connective tissue proliferation and appear in subsequent sections as papillomata. The papillomata rapidly become multiradicular; their branches unite with each other and with the branches of adjacent growths forming epithelial lined spaces resembling true acini. The walls of these spaces in turn give rise to papillary growths and there results an extremely complex structure comparable to that observed in human breasts and referred to as intracystic adenomata (Fig. 6). The tumors are not invasive at this stage and, with continued growth, the cyst or duct of origin is further expanded and the tumor may extend into all the radicles of the gland (Fig. 7). A point to be especially emphasized is the widespread nature of the process. Neoplasia is not localized as in the human disorder but usually occurs in separated regions throughout the mammary system.

Eventually, cellular changes resembling those associated with malignancy in man dominate the picture and occur in growths in widely separated regions. Lining cells varying in size and shape are arranged in multiple disordered layers. Nuclei are hyperchromatic, extremely irregular in structure and mitotic figures are common. The growths, however, remain confined within the walls of cysts or dilated ducts and their supporting stroma is not invaded. Occasionally, one observes extensions of growth into small ruptured areas in the walls of cysts containing large tumors but a connection with

the main tumor is always apparent and there is always a pronounced lymphocytic reaction in the surrounding tissues. Subsequent biopsies show no further evidence of invasion and for these reasons such extensions have been considered merely as the passive filling of defects created by the rupture of overdistended cyst walls and not as active invasion.

Stage of Invasion.—The duration of the disorder before the appearance of invasive changes varies greatly in individual animals but, eventually, microscopic examination shows multiple foci of invasion with no lymphocytic reaction. The released cells usually maintain the characteristics of the parent tumor and form abortive adenomatous structures in their new locations (Fig. 8) but in some instances further dedifferentiation apparently occurs, and the cells proliferate in solid, medullary sheets (Fig. 9). Early invasion is not limited to a single papillary growth or to a single breast but may occur widespread throughout the mammary tissue. The degree and rapidity of invasion, however, differ in different locations and it is a characteristic feature of the disease that rapidly progressive invasion in one breast is associated with slow less active invasion in other breasts.

The connective tissue response differs in individual animals and the growth rate and histological appearance of the tumor vary accordingly. Eventually, the entire breast is replaced by a scirrhous or medullary type of growth and the skin and muscle sheaths are extensively invaded.

Time Relations.—A determination of the duration of the various stages in the development of the disease is necessarily based on biopsy study, for definite clinical signs are not apparent until the changes which distinguish the advance of the condition have been present for some time. This entails examination of relatively small samples of mammary tissue obtained at more or less random intervals, and it is obvious that definite time relationships cannot be determined by the methods used in the present investigation. It is nevertheless apparent from a study of many cases that the length of the individual stages is extremely variable and bears no apparent relationship to the duration of the clinical course as a whole. The cystic stage may be very short and the stage of non-invasive neoplasia may be prolonged for more than a year while, in other cases, after a long period of simple cystic disease, papillomata pass rapidly into cancers. The epithelial cells of papillomata may show malignant changes for long periods of time without transgression of their normal boundaries, and in two instances such changes had been present without invasion for 4 and 6 months, respectively, when death occurred from unrelated causes. On the other hand, widespread invasion has been observed 2 months after the first appearance of cystic changes. The duration of life after the appearance of invasion varies from $1\frac{1}{2}$ to 11 months.

Metastasis.—Metastases have been found in 3 animals and in all of these the regional lymph nodes and the lung were involved. Metastases were

limited to these sites in one instance, while in another the mediastinal and retroperitoneal nodes also contained secondary growths. The third animal, on the other hand, showed a wide dissemination of metastases involving the pericardium, pleura, costal periosteum, liver, pancreas, kidneys, suprarenals, ovaries and bone marrow. It is of interest that the extent of metastatic dissemination bore a relationship to the length of the period of invasive growth in the primary tumor. The duration of this period was approximately 3 months in the first case, 8 months in the second and 11 months in the third.

Histologically, the primary growths in the first and third animals were medullary in type at the time of death and the metastases were generally similar in structure. A large thoracic tumor arising from the mediastinal nodes in the third animal, however, showed a more organized growth with the formation of acinus-like structures, many of which were widely dilated with a pale, eosinophilic secretion. The primary tumor in the second animal was adenomatous at the time of death and the metastases showed a comparable degree of differentiation (Fig. 10).

Tumors Not Associated with Preexisting Cystic Disease

During their earliest stages of development, the four tumors of this class showed a similar adenomatous structure consisting of numerous small but otherwise normal appearing acini divided by fibrous bands into large lobules. Sections from adjacent tissue and from other breasts showed a normal structure.

With a single exception, the morphological characteristics of the tumors in different animals remained similar with continued growth. The variation occurred as a consequence of a greater activity of acinar epithelium and it is significant that the animal bearing this tumor was of the same family group in which the previously described neoplasia of acinar epithelium occurred.

This animal died at the age of 50 months after the tumor had run a clinical course of 10 weeks. Microscopically, the acinar epithelium of one lobule of the growth had undergone extensive proliferation. Acini were widely dilated and filled with stratified layers of polyhedral cells. Many of the cells were heavily vacuolated and in some areas cell boundaries were indistinct or absent. Other lobules were made up of small, irregular acini lined by a single layer of epithelium. Mitotic figures were common but invasion was not observed (Fig. 11).

The acinar epithelium of the tumors in the remaining animals at no time exhibited the proliferation activity observed in the preceding case but, on

the other hand, remained confined to a single lining layer and growth proceeded through a multiplication of acini.

One animal died at the age of 46 months, a year after discovery of the tumor. Microscopically the greater part of the growth was made up of small, closely packed acini in atypical arrangement with frequent disorganized areas in which there was no attempt at acinar formation and the structure resembled that of carcinoma simplex. In other parts, the acini were distended with homogeneous eosinophilic material and occasionally formed macroscopic cysts (Fig. 12). There were no metastases.

Another closely related female died at the age of 5½ years, after the tumor had run a course of 16 months. The process in this instance did not remain localized to a single area and, while at autopsy the primary tumor had increased to a large mass, other smaller, rounded nodules of the same microscopic structure were found in other breasts. Histologically, the tumors were made up of irregularly formed acini lined by low, pale epithelium containing mitotic nuclei. The compact, circumscribed arrangement observed earlier in the primary nodule gave way to invasive growth after the 9th month and irregular strands and isolated groups of cells still in abortive acinar formation extended throughout the surrounding tissue (Fig. 13). At autopsy, further dedifferentiation with a complete loss of organized growth coupled with a pronounced connective tissue reaction resulted in a picture strikingly like that of scirrhous carcinoma in women. Metastatic tumors were found in the regional and mediastinal lymph nodes, the lungs, pleura, pericardium, diaphragm, spleen and right ovary. Histologically, the connective tissue reaction was less pronounced in the secondary growths but in other respects they resembled the primary tumor in appearance.

The fourth tumor of this nature was first noted at the age of 3½ years and its course was followed for 16 months until terminated by death with metastasis.

The first biopsy, undertaken 1 month after detection, showed a sharply circumscribed almond-sized nodule which on histological examination was found to be made of a compact mass of very small, acinus-like structures, some of which were elongated and slightly tortuous. The epithelium was relatively higher and the lumina less distinct than was observed in the previous tumor. In scattered areas acinar formation was poorly defined and mitotic figures were numerous (Fig. 14).

The tumor increased slowly in size for 10 months without any observed change in histological characteristics other than an increase in the number of mitotic figures. At this time a secondary nodule appeared in a breast of the opposite side and in the following 2 months grew to almost twice the size of the primary tumor. Histologically, the structure of this tumor was radically different from that of the primary growth and from the beginning manifested a complete lack of organization. There was no attempt at acinar formation and epithelial cells grew in solid, irregular masses and cords which infiltrated the connective tissue in all directions (Fig. 15).

Following the appearance of the secondary growth, the primary tumor showed no further increase in size but, at autopsy, presented areas of more anaplastic structure from which direct lymphatic extensions could be traced to the daughter growth. The skin and muscle in the vicinity of the secondary growth were extensively invaded at the

time of death and numerous isolated nodules were present in the surrounding tissue. Metastases of the same histological character were found in the lymph nodes of the axilla, groin, mediastinum and mesentery, and in the lung, diaphragm, liver, spleen, kidney, suprarenals, ovaries, cervix and vaginal wall (Figs. 16 and 17).

SUMMARY

The pathological histories of two types of familial mammary cancer in the rabbit have been described. One type was distinguished by characteristic antecedent mammary changes similar to those found in Schimmelbusch's disease in women and by a distinctive papillary structure. The second type originated in normal breast tissue and was characterized histologically by an atypical proliferation of acini.

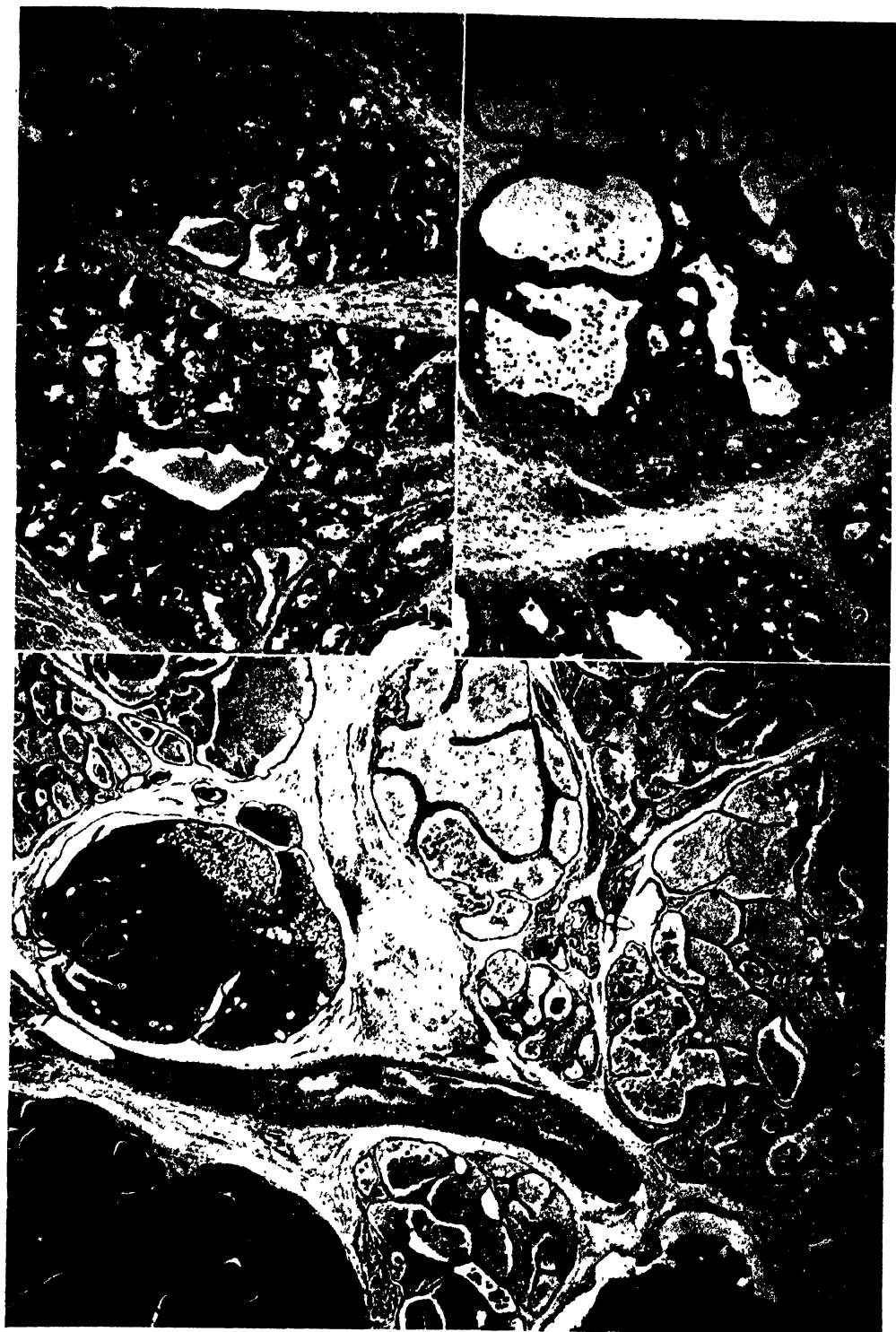
EXPLANATION OF PLATES

PLATE 13

FIG. 1. X10169-1. Section of breast tissue taken after recurrent periods of engorgement. Biopsy was performed after the peak of a phase of engorgement and the bulk of the gland was made up of clear, colorless fluid contained in thin-walled dilated spaces. The section was taken from a firmer area toward the periphery of a breast and shows hypertrophied gland fields. Many acini are dilated and distorted. Some contain an eosinophilic secretion. Hematoxylin and eosin. $\times 48$.

FIG. 2. BE75-6. Section of finely granular tissue which persisted after a phase of engorgement. The greater part of one gland field and segments of two others are shown. The intralobular connective tissue is condensed and is easily distinguished from the interlobular variety. A number of acini are widely dilated and contain desquamated cells. In one dilated acinus, the epithelium is arranged in multiple layers and is apparently in process of desquamation. Other acini are only slightly altered and some appear normal. About the periphery of the field acini are small and compressed. Hematoxylin and eosin. $\times 48$.

FIG. 3. B178. Section of breast tissue taken during the stage of cystic disease. A number of adjacent gland fields are shown. Both intralobular and interlobular connective tissue are reduced in amount and acini and gland fields lie in close proximity. All acini are distorted and widely dilated. Septal walls are thin and have ruptured in many regions so that acini intercommunicate. Whole gland fields have been converted into large cysts in this manner. Hematoxylin and eosin. $\times 20$.



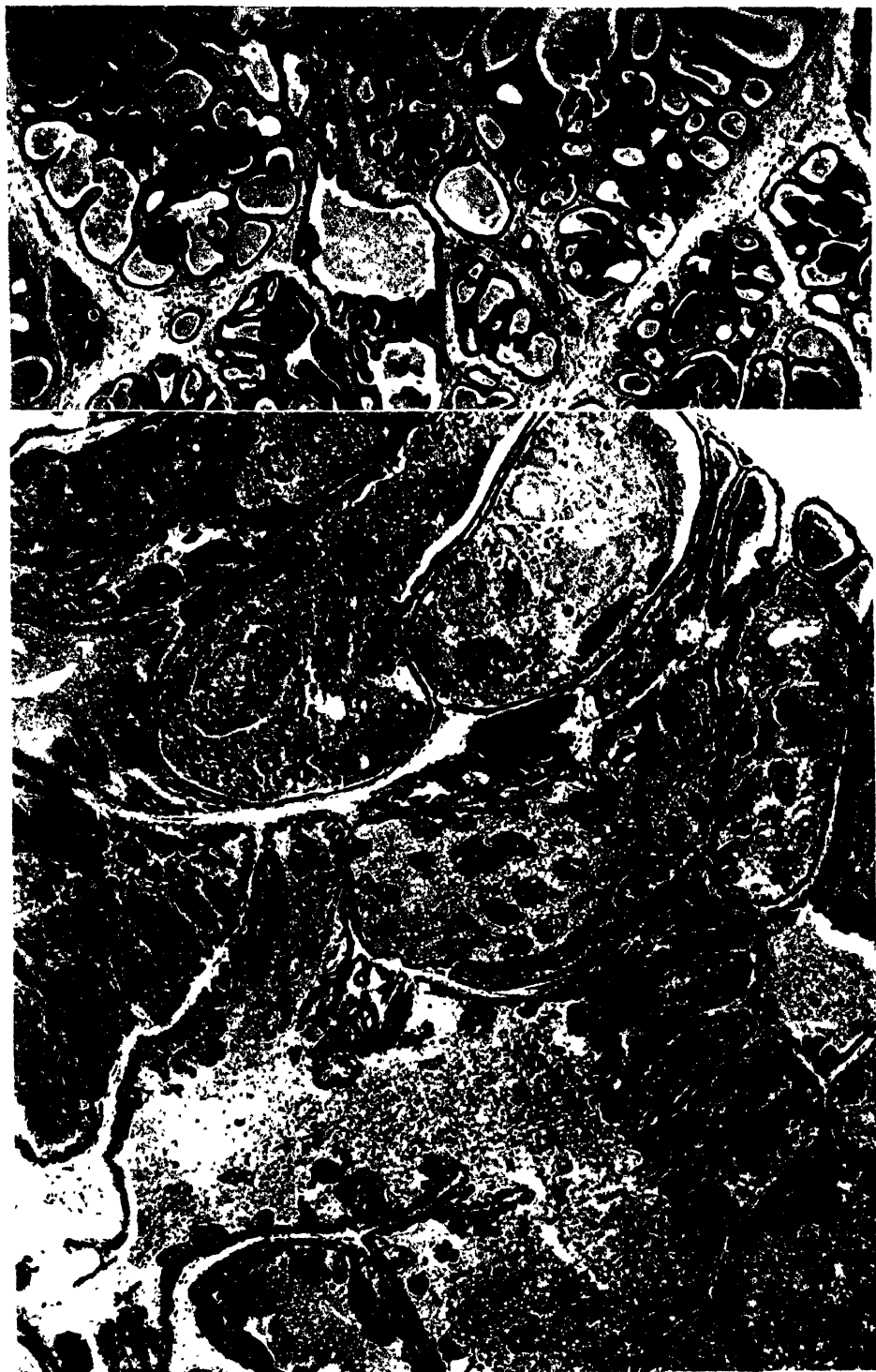
Photographed by J. A. Carlile

(Greene: Familial mammary tumors in rabbit. II)

PLATE 14

FIG. 4. X1966-4. Section of breast tissue showing numerous intra-acinar foci of epithelial neoplasia. In the majority of acini, the sessile growths are purely epithelial in character but, in some areas, a delicate connective tissue stalk can be made out. Hematoxylin and eosin. $\times 48$.

FIG. 5. B178. Section of breast tissue showing uniradicular papillomata arising from the epithelial lining of cyst walls. Compare with Fig. 3, which shows an earlier section from the same animal. Hematoxylin and eosin. $\times 48$.



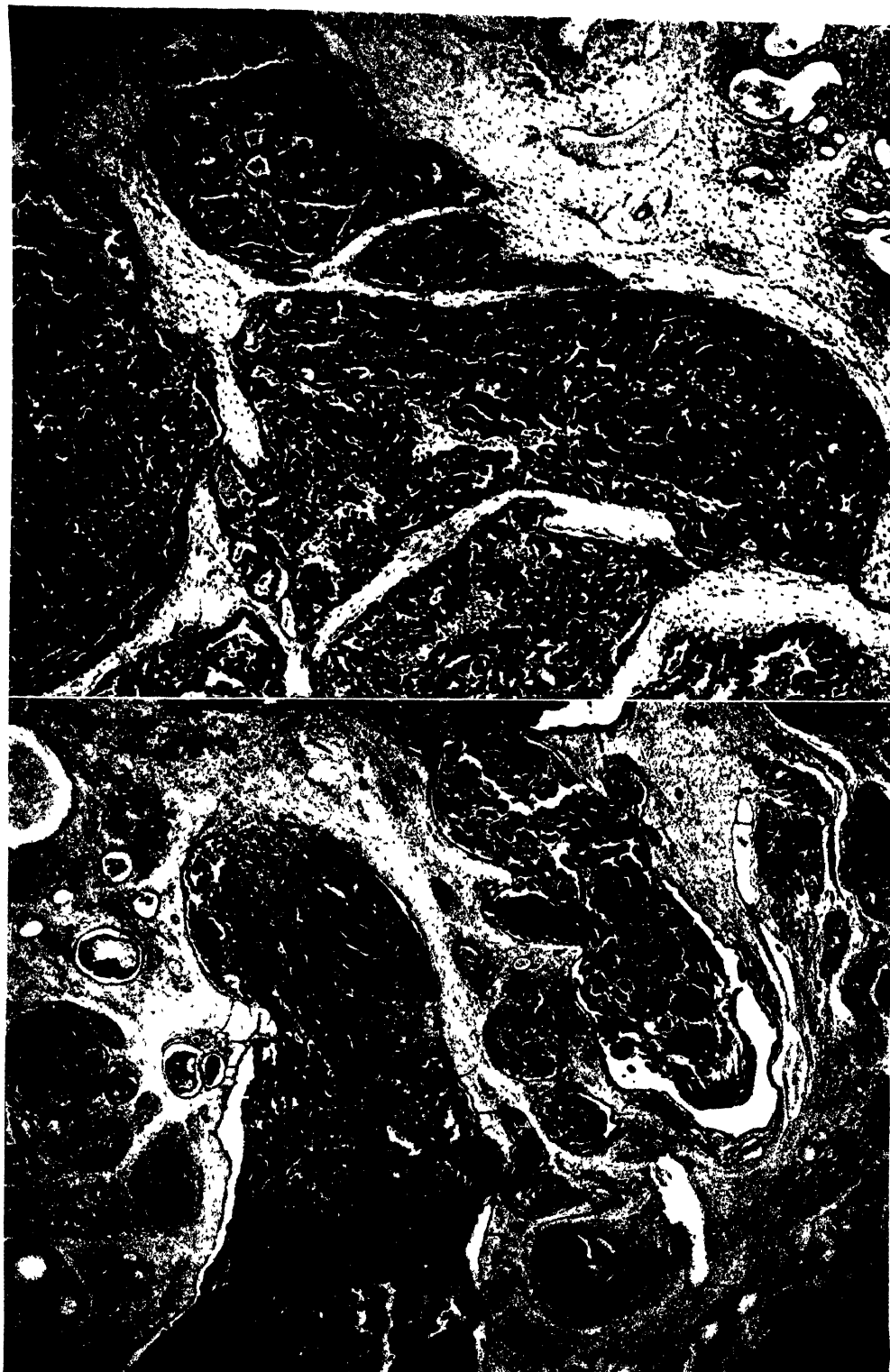
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(Greene: Familial mammary tumors in rabbit. 11)

PLATE 15

FIG. 6. B240-2. Section of breast tissue taken at a later stage in the development of neoplasia. The papillomata have become multiradicular and their branches have fused to form a complicated network of epithelial lined spaces. Hematoxylin and eosin. $\times 48$.

FIG. 7. B178. Section of breast tissue derived from the animal whose earlier biopsy sections are shown in Figs. 3 and 5. The growth has been forced into all the radicles of the duct system but, despite malignant cellular changes, invasion has not occurred. Hematoxylin and eosin. $\times 20$.



Photographed by J. A. Carlile

(Greene: Familial mammary tumors in rabbit. II)

PLATE 16

FIG. 8. B240-2. Section of breast tissue in an advanced stage of the disorder. Invasion has occurred and the released cells grow in an abortive papillary structure resembling that of the parent tumor. Compare with Fig. 6. Hematoxylin and eosin. $\times 48$.

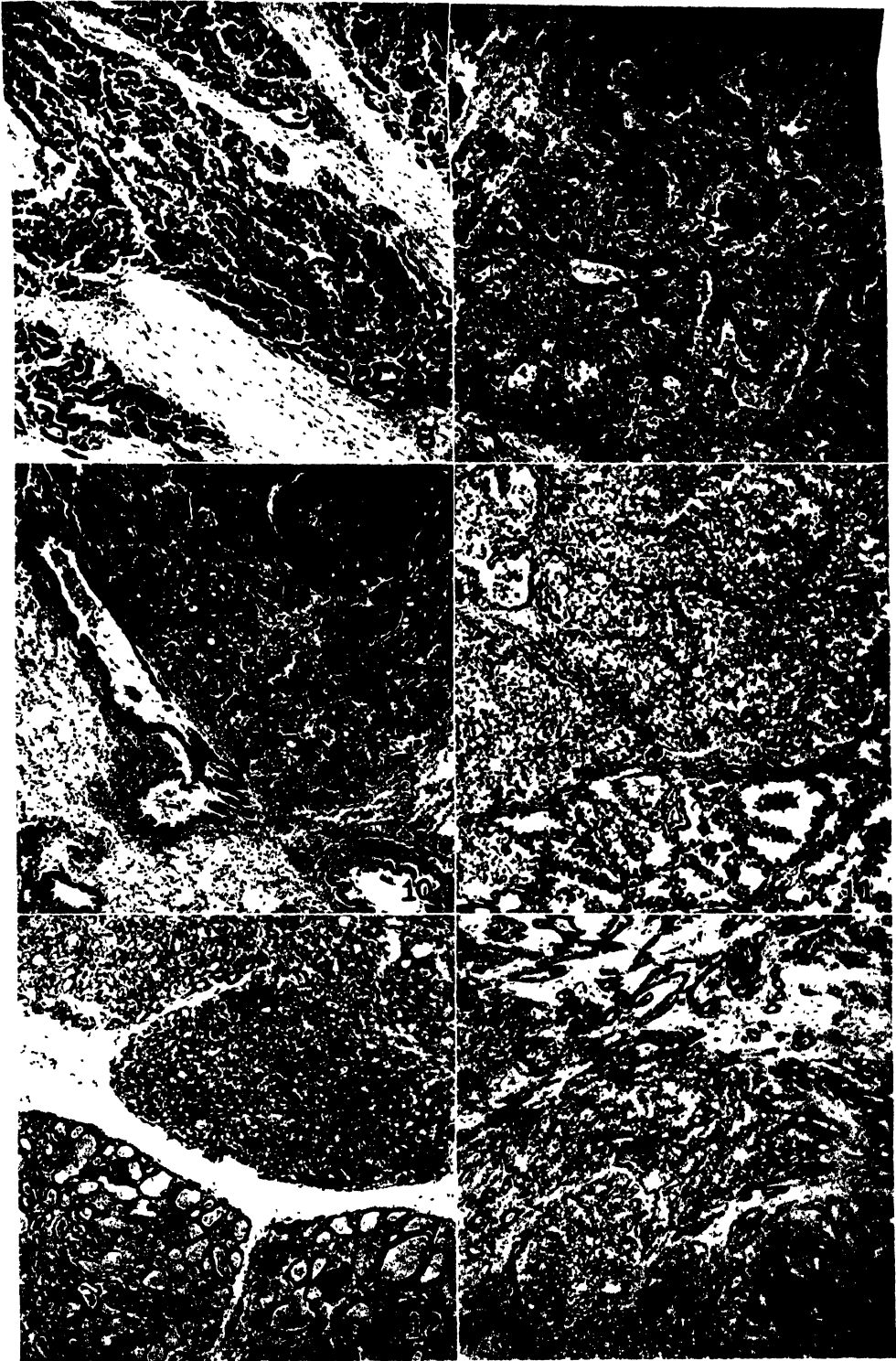
FIG. 9. B234-3. Section of a more anaplastic tumor. The neoplastic cells grow in solid medullary cords with no attempt at organization. Hematoxylin and eosin. $\times 48$.

FIG. 10. B240-2. Section of metastatic growth in the lung. Hematoxylin and eosin. $\times 45$.

FIG. 11. B57-2. Section of breast tumor of acinar type. The two types of structural growth which characterized this tumor are shown. The greater part of the tumor was structurally similar to the lower part of this figure and consisted of irregularly dilated acini lined by neoplastic epithelium. Other areas were similar to the upper portion of the figure and were distinguished by active epithelial proliferation. Hematoxylin and eosin. $\times 48$.

FIG. 12. E126-2. Section of breast tumor of acinar type. The bulk of the growth was made up of acinus-like structures containing an eosinophilic secretion. Other areas, as shown in the upper portion of the figure, were less organized in structure. Hematoxylin and eosin. $\times 48$.

FIG. 13. E33-5. Section of breast tumor of acinar type. The characteristic structural element was an irregularly formed acinus-like body lined by pale vacuolated epithelium containing numerous mitotic figures. The abortive acinar arrangement was preserved after invasion as is shown in the upper portion of the figure. Hematoxylin and eosin. $\times 48$.



Photographed by J. A. Carlile

(Greene: Familial mammary tumors in rabbit. II)

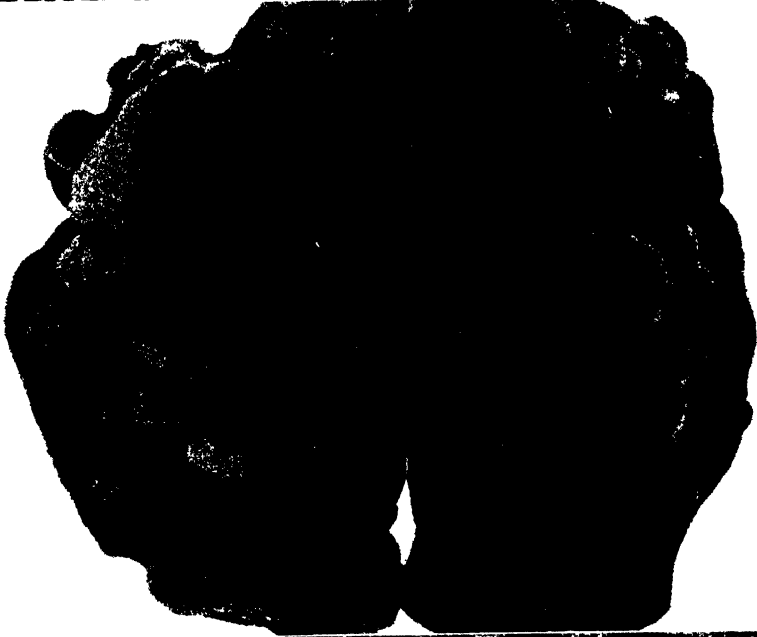
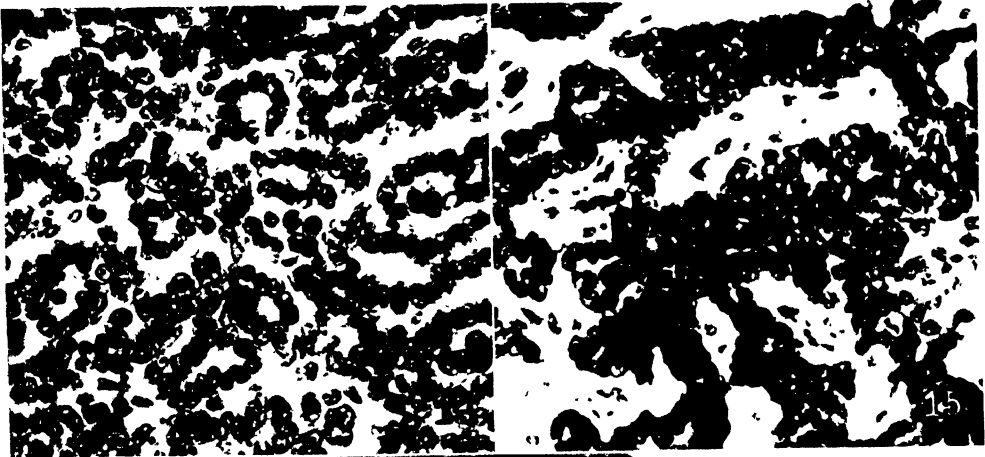
PLATE 17

FIG. 14. T36-1. Section of primary tumor taken shortly before death. This growth was characterized by a closely packed arrangement of rounded or slightly elongated acinus-like structures embedded in a minimum of stroma and showed little structural change throughout its course. Hematoxylin and eosin. $\times 310$.

FIG. 15. T36-1. Section of secondary tumor in an adjacent breast taken on the same day as the previous section. This growth from the beginning showed a total lack of organization and cells grew in solid infiltrating cords. Hematoxylin and eosin. $\times 310$.

FIG. 16. T36-1. Metastasis in the lungs and spleen. Despite the almost complete obliteration of lung tissue by secondary growths, death did not occur naturally and the animal had to be killed. Hematoxylin and eosin. $\times 118$.

FIG. 17. T36-1. Section of suprarenal showing the characteristic microscopic appearance of metastases. There is no connective tissue or other local reaction. The surrounding tissue is not compressed and growth was apparently invasive rather than expansive. Hematoxylin and eosin. $\times 40.5$.



16



Photographed by J. A. Carlile

(Greene: Familial mammary tumors in rabbit. II)

FAMILIAL MAMMARY TUMORS IN THE RABBIT

III. FACTORS CONCERNED IN THEIR GENESIS AND DEVELOPMENT

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PLATES 18 AND 19

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In the previous papers of this series which contain descriptions of the clinical and pathological course of spontaneous mammary tumors in the rabbit, attention was focused entirely on the local neoplastic process and no reference was made to the animals in which it occurred. A consideration of the tumors from the point of view of the animal as a whole rather than as isolated pathological occurrences brings out points of interest which bear particularly on the genesis of the growths. The object of this paper is to present the tumor-bearing animals from such a viewpoint, placing special emphasis on the occurrence of irregularities in physiological behavior and the presence of pathological changes in endocrine organs which tend to relate the spontaneous growths to certain types of experimentally induced tumors. In addition, the racial and familial relations of the tumor-bearing animals will be described and the findings reported in this and in previous papers will be discussed.

Heredity

An analysis of pedigree records shows that the mammary tumors of this series came almost exclusively from two family groups of rabbits, each of which presented a distinctive type of growth. The females of the two groups represented only a small part of the total female population of comparable age and only two of the many racial and family lines in the colony. For the most part these animals were bred with no knowledge of tumor potentialities. Moreover, in the present investigation no attempt was made to determine either the genetics of tumor animals or the precise rôle of inheritance. The material does not lend itself to such a study. However, the fact that breast tumors were limited to this small section of the population is presumptive evidence of the action of hereditary factors and, while it is not possible to draw final conclusions, there is evidence that such factors influenced both the character and course of the disorder.

For present purposes it is necessary to condense the data bearing on this aspect of the tumor problem. Representative pedigrees are given and other data are assembled in tabular form as a basis for the presentation of essential facts. Reference will be made to Tables I and II of the first paper of the series in this connection.

The origin and transmission of breast tumors in the Belgian line are shown in Chart 1 which gives the pedigree of B178. This and other pedigree charts are to be read from left to right. Thus, the parents of B178 were the female B16-2 and the male B14-2. In each instance the upper member of a pair is the female and the lower is the male; known tumor females are marked with an asterisk.

The Belgian stock of the colony came originally from the male 7459 and the female 7460 designated as the R strain. This female developed cysts of the breast with a suppurative mastitis and was killed, but the male and a son (498) by the above mentioned female were used extensively in the further development of the line and in crosses with four other strains of Belgians so that eventually the great majority of the Belgian population traced its ancestry to one or both of the original animals.

In the pedigree given in Chart 1, two other strains of Belgians are represented. One designated by the letter J was old Institute stock; the other, represented by the male 7954, was imported directly from England. Neither of these lines was used in the making of the hybrid crosses with other racial stocks from which breast tumors were obtained; these came largely from 498 and B14-2 on the one side and English rabbits on the other. It will be noted that B14-2 carries blood from the British as well as the R strain but pure Belgians and hybrids from the British stock have not developed tumors. In like manner, the J strain can be eliminated so that the R line remains as the only constant source of hereditary factors in the Belgian and Belgian hybrid population.

With the exception of the two hybrids X1539-1 and X2594-4, every animal listed in Table I, Paper I, was derived on one or both sides from the male 7459 or from both 7459 and 7460. Moreover, as shown in Chart 1, breast tumors have been found in the daughters of tumor mothers for as many as three successive generations, 523-2, B16-2, B178. In the case of the hybrids mentioned, both were mongrels with blood from several racial lines including English but not Belgian. The group as a whole, therefore, is either Belgian or English or both with blood from other racial lines in some instances. This third element, however, is a widespread variant.

The source of the hereditary factors on the English side of the Belgian-English hybrids and the pure English listed in Table II, Paper I, is less certain. If the existence of an English tumor line is assumed, for which there is some evidence, an analysis of pedigrees shows that the two English and the English ancestor of the English-Tan female came from a single family line which goes back on both sides to the male J5 and one of two females, 7596 or J, as shown in Chart 2. These were foundation stocks and the line is known as our dwarf English line. It was developed from J5 and 7596, through the male 443-3 which in reality became the head of the line.

As in the case of the Belgians, it was impossible to maintain this line in pure form and it was outcrossed from time to time with another English strain. This strain is represented in the pedigree chart by the male 7944, the head of the line.

The English stock used for hybridization with the Belgians came largely from the

strain represented by the male 7944 and a female 7597 as shown in Chart 3. Of the 18 hybrids listed in Table I, Paper I, only 3 contained blood from the dwarf English or breast tumor line (Chart 4). These were BE44-2, X6106-3 and X2594-3; 2 of these had rapidly progressive cancerous lesions as compared with 2 cases of cancer among the 15 animals derived from the other English line.

There is, however, another factor which must be considered in this connection, namely, the relative preponderance of Belgian or English blood and its probable influence. This

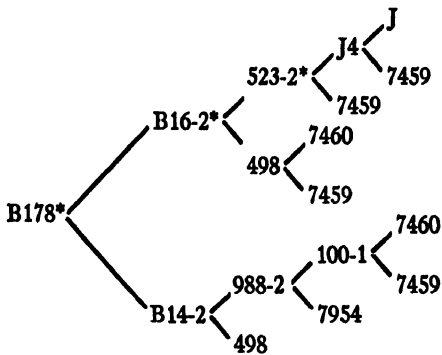


CHART 1

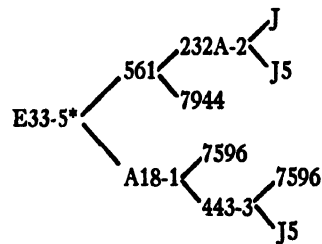


CHART 2

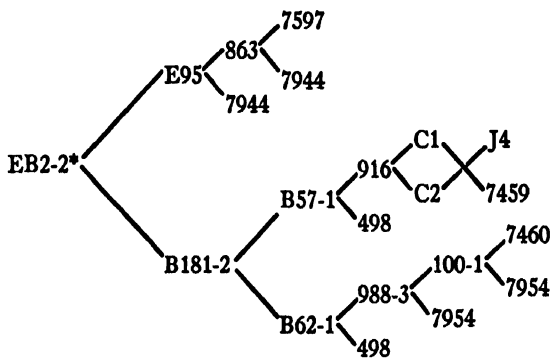


CHART 3

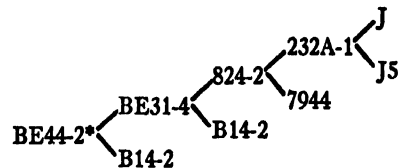


CHART 4

is best brought out by a consideration of apparent differences between Belgian and English tumors on the one hand and the tumors of hybrid stocks on the other.

It will be recalled that the division of material contained in Tables I and II, Paper I, was based on the character of the initial pathological process. Table II contains 4 animals of which 3 were English, one an English-Tan hybrid and one a Belgian. An examination of this table shows that the 3 cases of cancer occurred comparatively late in life and in 2 instances death occurred at 67 and 58 months respectively. The third animal was still in excellent condition when killed at the age of 46 months.

In Table I, it will be noted that there were 4 pure Belgians with cancerous lesions. The age of onset varied between 25 and 30 months, while the age at death of the 3 that died or were moribund when killed was 37 to 54 months. 2 other animals, B348-1 and BE44-2 may be added to this group. These animals were derived from a Belgian-English

cross followed by a backcross to the Belgian line and data on their tumors place them in the Belgian rather than in the English group. One other animal in Table I, X6106-3, is of interest in this connection. This animal was an F₁ Belgian hybrid derived from a cross between a Belgian female and an F₁ English-Polish male, the English blood coming in this case from the English tumor line. The animal was the only one of this particular class in the breast tumor group and also conformed with the Belgian type of behavior. It should be noted in this connection that of the three Belgian-English hybrids mentioned in this paragraph, two were crosses between the two tumor lines and, while one was an F₁ Belgian hybrid and the other a backcross Belgian, yet their behavior was essentially the same.

The hybrids in the lower section of Table I, from EB2-2 to X10557-3 inclusive, are of particular interest. It will be noted that taken as a group, the mammary changes either developed relatively late in life or progressed slowly in comparison with the pure Belgians and other animals referred to above. In the case of EB2-2, the breast tumor has been morphologically malignant for the past 9 months but the animal is still breeding. It is now 58 months old and has exceeded the period of survival usually observed in the Belgians. The behavior in this respect is more in line with the English tumor group. This rabbit is an F₁ English-Belgian cross (English mother and Belgian father); BE75-6 is also an F₁ Belgian-English cross of the reverse order, while the other animals of this group, with the exception of X10169-2, are predominantly English. X10169-2 is an animal of an entirely different genetic classification and can hardly be considered in the present connection.

The facts pointed out above have been reduced to tabular form and are presented in Table I of the present paper. It will be observed that the terminal condition in the Belgians and in the Belgian-English hybrids with predominant Belgian blood was cancerous in the majority of cases. On the other hand, with a single exception, the disorder in the hybrids of predominant English blood has not yet reached a malignant stage despite a comparable duration. The three groups are small but the behavior of the animals in the different classifications tends to be consistent. The characteristics of the breast tumors in the Belgian population and the Belgian-English hybrids derived from a backcross to the Belgian line as well as those derived from a cross between the two tumor lines tend, as far as they are known, to conform to one pattern with respect to age relations as well as to pathogenesis. The case of B57-2 (Table II, Paper I) is a notable exception to this rule. This animal was the mother of B240-2 (Table I, Paper I) and the situation presented by these two animals indicates that while, for unknown reasons, a given animal may deviate from the usual pattern of behavior, the progeny of such an animal may act in conformity with the family group rather than with the immediate parent.

The Belgian-English hybrids in which the English blood predominates conform to the Belgian rather than the English tumor type. However, the

tumor age relations in this group which indicate either a delay in the initiation of the disorder or a retardation in its progress as compared with tumor development in the other groups tend in the direction of the English tumor line (Table II, Paper I).

While there can be no doubt concerning the action of hereditary factors in the production of these tumors, available evidence as to the mechanism of

TABLE I
The Influence of Predominant Belgian or English Blood on Tumor Age Relations

Classification	No.	Age at inception of disorder	Duration of disorder	Age at present or at death	Terminal or present condition
		<i>mos.</i>	<i>mos.</i>	<i>mos.</i>	
Belgian	B16-2	29	25	54	Carcinoma
	B178	25	19	44	"
	B234-3	30	7	37	"
	B240-2	29	16	45	"
	B346-3	27	13	40	Papilloma
Belgian-English*	B348-1	20	7	27	Carcinoma
	BE44-2	29	2	31	"
	X6106-3	17	21	38	"
Belgian-English†	EB2-2	29	29	58	Carcinoma
	BE75-6	43	4	47	Epithelial neoplasia
	X5943-9	44	3	47	Papilloma
	X7634-6	33	3	36	Cystic disease
	X7768-3	30	6	36	" "
	X8157-1	19	16	35	Papilloma
	X10557-3	10	16	26	Cystic disease

* Predominantly Belgian. BE44-2 and X6106-3 also represent crosses between the tumor lines.

† Predominantly English or F₁ Belgian-English.

inheritance is contradictory. An analysis of pedigrees shows that in some instances breast tumors have developed in first generation crosses between Belgian males and unrelated females as well as in crosses of the reverse order. This, however, is the exception rather than the rule. In fact, the incidence of tumors even among the daughters of tumor mothers by males known to be capable of transmission has been very low, although, as has been shown, there are instances of a mother to daughter transmission for two to three successive generations. Expression of the tendency is therefore recessive as a rule but occasionally it appears to be dominant. This paradoxical situation would indicate that the hereditary influence is not of a simple

TABLE II
Reproductive History of Tumor-Bearing Animals

No.	Breeding period		Duration	Number of matings	Fertility	Normal delivery	Abortion	Resorption of products of conception	Average number of young in litter	Dead-born young
			mos.		per cent	per cent	per cent	per cent		per cent
B16-2	Before inception of disorder	1st half	12	6	66.6	50	50	0	4.0	25.0
		2nd half	12	5	80.0	100	0	0	2.7	18.1
	During disorder		11	11	27.2	100	0	0	4.3	7.6
B178	Before inception of disorder	1st half	11	8	62.5	100	0	0	3.8	5.2
		2nd half	11	11	9.0	100	0	0	?	
	During disorder		12	13	0					
B234-3	Before inception of disorder	1st half	12	12	25.0	100	0	0	5.0	0
		2nd half	12	13	15.3	0	0	100		
	During disorder		7	9	11.1	100	0	0	2.0	0
B240-2	Before inception of disorder	1st half	11.5	10	30.0	100	0	0	3.6	0
		2nd half	11.5	8	12.5	100	0	0	6.0	0
	During disorder		15.0	17	29.4	100	0	0	4.8	8.3
B346-3	Before inception of disorder	1st half	9.0	5	60.0	100	0	0	5.6	11.7
		2nd half	9.0	5	60.0	100	0	0	7.0	4.7
	During disorder		8.0	6	16.6	100	0	0	7.0	0
B348-1	Before inception of disorder	1st half	5.5	3	66.6	100	0	0	7.5	6.6
		2nd half	5.5	4	25.0	100	0	0	6.0	0
	During disorder		6.0	4	25.0	100	0	0	2.0	0
BE28-3	Before inception of disorder	1st half	15.0	5	80.0	100	0	0	7.5	0
		2nd half	15.0	7	14.2	100	0	0	5.0	0
	During disorder		1.0	1	100.0	0	0	100		
BE44-2	Before inception of disorder	1st half	11.5	8	37.5	100	0	0	2.6	50.0
		2nd half	11.5	12	8.3	0	0	100		
	During disorder		2.0	6	0					
X1966-4	Before inception of disorder	1st half	15.5	6	100.0	100	0	0	8.3	0
		2nd half	15.5	7	71.4	100	0	0	8.2	0
	During disorder		6.0	4	25.0	0	0	100		
X5074-2	Before inception of disorder	1st half	8.5	7	42.8	100	0	0	6.6	0
		2nd half	8.5	4	75.0	100	0	0	5.0	0
	During disorder		0	0						
X6106-3	Before inception of disorder	1st half	5.5	4	75.0	100	0	0	7.6	43.4
		2nd half	5.5	3	66.6	100	0	0	4.5	44.4
	During disorder		19.0	17	47.0	87.5	0	12.5	5.9	22.5

TABLE II—*Continued*

No.	Breeding period		Duration	Number of matings	Fertility	Normal delivery	Abortion	Resorption of products of conception	Average number of young in litter	Dead-born young
			mos.		per cent	per cent	per cent	per cent		per cent
X9755-3	Before inception of disorder	1st half	3.0	1	100.0	100	0	0	6.0	16.6
		2nd half	3.0	1	100.0	100	0	0	4.0	100.0
	During disorder		7.0	4	50.0	100	0	0	6.5	15.3
X2594-3	Before inception of disorder	1st half	14	9	55.5	100	0	0	5.8	0
		2nd half	14	11	54.5	100	0	0	5.3	0
	During disorder		0	0						
X1984-4	Before inception of disorder	1st half	18	11	45.4	100	0	0	6.8	23.5
		2nd half	18	3	100.0	100	0	0	4.0	25.0
	During disorder		2	4	0					
X1539-1	Before inception of disorder	1st half	15.5	10	50.0	100	0	0	6.4	0
		2nd half	15.5	7	0					
	During disorder		4.0	2	0					
EB2-2	Before inception of disorder	1st half	12.0	4	100.0	100	0	0	6.5	3.8
		2nd half	12.0	3	100.0	66.7	0	33.3	4.5	22.2
	During disorder		29.0	11	63.6	57.1	14.3	28.6	2.7	45.4
BE75-6	Before inception of disorder	1st half	18.0	8	75.0	100	0	0	6.3	10.5
		2nd half	18.0	6	83.4	80	20	0	2.7	45.4
	During disorder		4.0	3	66.6	100	0	0	1.0	100.0
X5943-9	Before inception of disorder	1st half	18.0	9	66.6	100	0	0	9.3	10.7
		2nd half	18.0	6	100.0	100	0	0	5.6	14.7
	During disorder		3.0	2	50.0	100	0	0	7.0	0
X7634-6	Before inception of disorder	1st half	13.5	4	100.0	100	0	0	5.7	0
		2nd half	13.5	3	100.0	100	0	0	7.0	0
	During disorder		3.0	2	50.0	100	0	0	6.0	0
X7768-3	Before inception of disorder	1st half	12.0	5	80.0	100	0	0	7.5	13.3
		2nd half	12.0	6	83.3	80	20	0	5.5	0
	During disorder		6.0	5	60.0	100	0	0	5.3	0
X8157-1	Before inception of disorder	1st half	6.5	3	66.6	100	0	0	8.5	0
		2nd half	6.5	2	0					
	During disorder		16.0	12	25.0	66.7	33.3	0	5.5	54.5
X10169-1	Before inception of disorder	1st half	3.0	1	0					
		2nd half	3.0	2	0					
	During disorder		15.0	14	35.7	0	0	100		

TABLE II—*Concluded*

No.	Breeding period		Duration	Number of matings	Fertility	Normal delivery	Abortion	Resorption of products of conception	Average number of young in litter	Dead-born young
			mos.		per cent	per cent	per cent	per cent		per cent
X10557-3	Before inception of disorder	1st half	0	0						
		2nd half	0	0						
	During disorder		16.0	7	57.1	100	0	0	6.2	12.0
B57-2	Before inception of disorder	1st half	16.5	9	55.5	100	0	0	3.4	5.8
		2nd half	16.5	8	62.5	100	0	0	4.0	0
	During disorder		0	0						
E33-5	Before inception of disorder	1st half	21.5	8	75.0	100	0	0	7.6	17.4
		2nd half	21.5	11	36.3	100	0	0	5.5	31.8
	During disorder		8.0	2	0					
E126-2	Before inception of disorder	1st half	12.0	9	66.6	100	0	0	4.8	0
		2nd half	12.0	10	10.0	100	0	0	3.0	33.3
	During disorder		0	0						
T36-1	Before inception of disorder	1st half	10.5	5	20.0	100	0	0	3.0	0
		2nd half	10.5	8	0					
	During disorder		0	0						

Mendelian order or that factors other than heredity play a decisive rôle in determining the expression of hereditary characters. Evidence obtained from pedigrees is not sufficient in itself to clarify this situation.

Numerous animals of unrelated lines have been fostered by tumor-bearing animals and have been held under observation for long periods of time but in no instance have mammary tumors developed. This, together with the low incidence of tumors among the daughters of tumor mothers is evidence against the passage in the milk of a factor influencing tumor development.

Reproductive History

Essential facts relating to the reproductive history of 23 tumor-bearing animals and of 4 animals with cystic disease are recorded in Table II.

The table is arranged to contrast the results of breeding activity during the period of disorder and the period previous to its inception and, for this reason, data concerning 2 tumor-bearing animals in which the duration of the condition was not determined are omitted. The breeding period preceding the clinical discovery of mammary changes is

divided into equal halves to bring out existing differences and the first half of the period is used as a standard for purposes of comparison. It should be noted in this connection that in normal animals of similar age groups, the second half of a comparable period is almost always characterized by an improvement in breeding records. 21 of the 27 animals under consideration were bred during the various intervals defined in this manner and pertinent data are available.

Fertility.—The fertility rate was reduced during the period of mammary disorder in 90.4 per cent of the affected animals. 5 of the animals were completely sterile and in all but one of the remaining instances the reduction was of significant proportions. On the other hand, the percentage of fertile matings was increased in 2 cases, but in both the number of matings was insufficient to allow an accurate comparison of the different intervals and the findings are without significance.

Fertility was likewise reduced, in the majority of cases, during the latter half of the period preceding mammary changes. In a number of instances, the rate was unchanged or increased but, in all such cases, a division of the period into smaller intervals showed a gradual increase in the number of sterile matings.

As a rule, the proportion of infertile matings was progressive and reached a low point during the course of disorder, but in a number of instances sterility was more pronounced in the period immediately preceding the appearance of mammary changes and was followed by improvement with increased fertility which approached or exceeded that of the first interval.

Gestation.—17 of the listed animals proved fertile during the course of the disorder and in 11, or 58.8 per cent of them, the various gestation periods proceeded normally and parturition occurred at the end of a usual term. In the 6 remaining cases, however, one or more of the gestation periods terminated prematurely in abortion or in resorption of feti.

Abortion occurred in a single animal during the first interval before inception of the disorder but, on the other hand, either abortion or resorption of feti terminated gestation periods in 22.7 per cent of the 22 animals that became pregnant during the second interval.

Litter Size and Dead-Born Young.—The average number of progeny was reduced in the litters of 7 of the 12 animals that bore young during the period of clinical disorder. A progressive reduction in average litter size throughout the period of breeding was a rare finding but a general decrease occurred during the interval preceding breast changes and involved the litters of 77.7 per cent of the animals that gave birth at that time.

The incidence of dead-born young in litters was increased during the

disorder in 33.3 per cent of the animals. This manifestation was also apparent in the preceding interval during which it was more generalized and involved 44.4 per cent of the animals.

Group Average.—A consideration of the group as a whole rather than of individual animals emphasizes the variation in reproductive function that characterized breeding history in successive clinical periods. Table III is constructed from this point of view and presents the results obtained from averaging the activity of the entire group during the various intervals defined in a previous paragraph. All of the different indices of reproductive function used in this analysis give evidence of a progressive disturbance which preceded the appearance of mammary changes and reached a peak during tumor development.

TABLE III
Summary of Reproductive History of Tumor-Bearing Animals

Breeding period		Fertility	Normal delivery	Abortion	Resorption of products of conception	Average number of young in litter	Dead-born young
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>
Before inception of disorder	1st half	58.2	98.0	2.0	0	6.2	8.9
	2nd half	39.5	91.0	3.0	6.0	5.2	10.8
During disorder		31.4	75.6	4.0	20.4	4.8	17.3

Maternal Care.—An exact analysis of alterations in maternal care is not possible from the available data, for in many cases litters were discarded or fostered at birth. It was apparent, however, that in the majority of cases no great variations were related to the occurrence of the disorder and that the tendencies expressed during early breeding history continued to characterize the animal throughout its reproductive career.

As a rule, if an animal manifested a disposition to desertion or poor maternal care at an early period, this tendency became more pronounced with continued breeding. However, outstanding exceptions to this generalization occurred and are typified by the history of one rabbit, B240-2. In this instance, the animal had been an exceptionally poor breeder and had failed to raise a single litter but became an excellent breeder with a relatively high fertility percentage and succeeded in raising thrifty young even after biopsy sections of the breast mass had shown invasive growth. There were other cases of this kind but the above is of particular interest inasmuch as the improvement in reproductive function was accompanied by other evidences of constitutional change. The animal had been fat, disinclined to activity and generally of poor type, but following

the occurrence of breast changes a profound transformation became apparent with loss of fat and lassitude and a reversion to the slender, racy, Belgian conformation.

General Pathology of Tumor-Bearing Animals

While the dominating gross lesion in these animals was the mammary tumor, pathological changes found in other organs at autopsy reflected the physiological disturbances observed clinically and were of such a nature as to suggest that the presence of neoplasia in the breast formed only a local manifestation of a generalized constitutional disorder. The pituitary and suprarenal glands of animals in the various stages of tumor development showed consistent and unusual alterations. The thyroid and uterus also presented abnormalities which, however, are found in other disorders throughout the general animal population.

Animals with an Antecedent History of Cystic Disease.—The suprarenal changes were present in animals killed during the phase of cystic disease but were usually more pronounced in subsequent stages (Fig. 1). They consisted in the presence of small, irregularly rounded, pale, grey areas which in routine sections appeared to be made up of clusters of large, washed-out cells containing pyknotic nuclei and numerous small vacuoles. Such areas were found scattered throughout the cortex or medulla but tended to be concentrated in the inner portion of the zona reticularis and their presence was indicated in the gross by a distinct yellowish ring in this region. In later stages, the nuclei disappeared and cell boundaries became indistinct so that, except for the vacuoles which enlarged and coalesced, the areas appeared more or less homogeneous.

The pituitary was enlarged in all stages of the disorder (Fig. 2) and a comparison of the weights of glands obtained from animals killed at different periods indicates a relationship between the degree of enlargement and the progress of the disease. The weight of glands obtained during the cystic stage averaged approximately one and one-half that of pituitaries derived from normal animals of the same breed, age and size. Using the same standard of comparison, the glands increased 2 to 3 times during the stage of non-invasive neoplasia and from 4 to 5 times after active invasion. The largest gland observed in the present series weighed 308 mg. which represents a fivefold increase based on an average normal weight of 60 mg.

The enlargement was found on section to be due almost entirely to hyperplasia of the chromophobic cells of the anterior lobe. In the great majority of cases, the hyperplasia occurred widespread throughout the lobe, but in a few cases it remained localized with the production of adenoma-like masses (Fig. 3). Granular cells, which were represented chiefly by acidophiles, were more numerous in cases of localized chromophobic hyperplasia but were greatly diminished in both types. Their reduction was least marked in animals killed during the cystic stage but was so pronounced in animals with invasive tumors that whole sections failed to show a single granular element.

Cells of the intermediate lobe were often found deep in the substance of the anterior, as well as of the posterior lobe, but the frequency of this finding in apparently normal animals renders it of doubtful pathological significance. So called Herring bodies were

unusually distinct and numerous in the posterior lobe and large masses of similar staining material occupied variable portions of the intermedia border.

The follicles of the thyroid were widely dilated with pale eosinophilic material and were lined by flattened epithelium. Septal walls were ruptured in scattered areas producing cysts which were occasionally detected macroscopically. The stroma of the gland was hyaline in appearance and appeared infiltrated with material possessing the staining qualities of colloid.

The endometrium of the uterus was hyperplastic and gross cysts were common (Fig. 4). In the majority of cases, the hyperplasia was glandular in type and sections showed the typical Swiss cheese pattern observed in the disorder in women. Occasionally, the hyperplasia was limited to the stroma, glands were absent and the lining epithelium atrophic.

Additional foci of neoplasia were found at autopsy in 3 animals with cancer and in 3 with non-invasive anastomosing papillomata. The coincident tumors in the second group were all adenomata of the uterus, 2 of which had invaded the muscular coat and were definitely carcinomatous. A similar growth was found in one animal of the first group, a uterine hemangioma in another and a carcinoma of the skin of the foot with metastasis to a popliteal lymph node in a third.

Animals with No Abnormal Antecedent Breast History.—With the exception of the pituitary, the endocrine organs of animals bearing tumors of this type showed pathological changes comparable to those found in the animals described in the previous section. The pituitary glands, on the other hand, were either normal in size or only moderately enlarged and exhibited no gross disproportion in granular and non-granular elements. The endometrium in all instances showed pronounced cystic hyperplasia and, in one case, was also involved in an invasive adenomatous growth. The ovaries and parovarian tissue frequently contained pea-sized cysts and the right ovary of one animal was the site of a large cyst containing 50 cc. of fluid.

Circumstances Influencing the Course of Tumor Development

It is apparent from examination of Table I of Paper I that the duration of the disorder and of its individual stages was subject to considerable variation. The same anatomical changes were frequently found in the breasts of animals killed after periods of disease of extremely variable duration. In addition, invasion was present after a course of 2 months in one instance, while in another the disorder had not progressed beyond the stage of cystic disease after a duration of 16 months.

Such variations constitute an integral part of the investigation of these tumors and this phase of the study is being developed at the present time. The material now available is unsuitable in many ways for statistical analysis but certain observations relative to the influence of different factors on the course of the disorder will be considered in the present section both because of the implications involved and because they indicate the trend of future studies.

Age.—The progress of the disorder was slow and abortive in a number of

the older animals of this series but there is no definite evidence to indicate whether age or a coincident factor was of significance in this respect. It is apparent, however, that an age difference distinguished animals bearing tumors of the different types. Cystic changes appeared in the breasts of one group at ages varying from 10 to 44 months while tumors were discovered in animals without antecedent cystic disease at ages ranging from 34 to 51 months. The average age was 28.3 months in the first group and 43.5 months in the second.

Breeding History.—Reproductive activity was of no apparent influence on the course of the disorder after the development of neoplasia. It was constantly observed, however, that the stage of cystic disease was considerably prolonged if affected animals remained fertile and were allowed to nurse their litters. In such cases, pronounced cystic changes present before pregnancy frequently disappeared entirely after lactation or only a few areas of granular thickening persisted.

Physical Condition.—The general health of the animal played an appreciable rôle in the course of both cystic and neoplastic stages. As a rule, if the physical condition became impaired, the sequence of events proceeded slowly or the disorder was arrested for long periods of time in any one of its developmental stages. Arrested development of this nature was particularly noticeable during the early phases of cystic disease and, in a number of instances, the breasts of animals in poor health remained in a state of partial engorgement with minor cystic changes for more than a year. Regression of existing changes accompanied impairment of general health in other cases.

In one specific instance, an animal with pronounced cystic disease suffered partial paralysis and a sudden deterioration in physical condition following parturition. All of the litter died shortly after birth and the doe was not nursed. The cystic breast lesions underwent coincident involution until the presence of abnormal changes could only be established by careful examination. Eventually the paralysis disappeared and the animal returned to health. Simultaneously, cystic disease recurred and the disorder passed rapidly into the stage of papillary neoplasia.

Coincident Neoplasia.—It was noted in the previous paper that the presence of rapidly invasive growth in one breast was associated with slow, less active invasion in the remainder of the mammary system. A restraining influence of the same nature was also apparent in unrelated neoplastic lesions in other organs, and in a number of cases, the action tended in a reverse direction and breast tumors with pronounced anaplastic cellular changes remained confined to normal boundaries in the presence of an invasive tumor in another region of the body.

A reciprocal relationship of this character was evident in 5 of the 6 cases in which coincident neoplasia existed. In one instance (B16-2) a coexisting hemangioma of the uterus showed slow growth and little histological evidence of activity. In another instance (B234-3) an endometrial adenoma of known duration remained confined to the mucosa without the muscular invasion that characterized ordinary tumors of this type after a similar period of development. A reversal of this relationship was apparent in other animals. In 2 cases (X1984-4 and X1539-1), the secondary focus of neoplasia in the uterus advanced to cancer while the breast disorder remained in an early developmental stage for a long period of time. In a third instance (B346-3) the presence of an epithelioma of the skin of the foot with a metastatic growth in a popliteal lymph node was associated with long continued anaplastic cellular changes without invasion in the mammary tumor. On the other hand, the growths in the breast and in the uterus advanced at a comparable rate in one animal (E33-5) and, while death was eventually caused by metastases from the mammary tumor, wide, local invasion was present in the uterus.

Cystic Disease without Subsequent Neoplasia.—The position of cystic disease as an essential stage in the development of one type of neoplasia is beyond question, but a type of cystic disease which is not followed by new growth is of occasional occurrence in the general rabbit population. The mammary changes in this type are confined to small, isolated, pea-sized cysts. The disorder is not progressive and, on the contrary, tends to disappear with time. Cystic disease of the type described in a previous section and characterized by large, multiple cysts has never been observed as an isolated disorder, but if the animal lives is always progressive in character.

Animals with uterine adenomata frequently show cystic changes which may be widespread throughout the mammary tissue. The disease may persist with periods of exacerbation and regression throughout the life of the animal. In the great majority of cases, the changes do not progress beyond the cystic stage, but in a number of instances, as previously noted, neoplasia in the breast dominated the picture.

DISCUSSION

An investigation of mammary tumors in the rabbit brings out two main points which warrant further consideration. The occurrence of two types of neoplasia in the rabbit, one of which originates in breasts which have been the seat of cystic disease and the other in tissue without antecedent clinical change, would seem to bear directly on a problem in human medicine. The existence of specific endocrine changes in both types, on the other hand, is of interest to experimental cancer research inasmuch as similar changes are found in animals subjected to treatment with one class of carcinogenic substances.

The relationship between cystic disease of the breast and the develop-

ment of cancer is a much debated question in human pathology. The majority opinion favors the existence of a definite relationship, the nature of which, however, remains controversial. One school holds cystic mastitis to be itself a neoplastic disease and considers progress to cancer comparable with the anaplastic advancement of other tumors. Other workers, with varying conceptions of the fundamental nature of cystic disease, stress the importance of abnormal environmental relations imposed on mammary epithelial cells during cystic disease in their subsequent neoplastic transformation. On the other hand, a large group of observers emphatically deny the existence of any association between cancer and cystic mastitis and some maintain a purely inflammatory conception of the origin of the latter.

All of the rabbits in which cystic disease was followed by neoplasia were members of a genetically related group but, outside this family, cystic disease did not progress to neoplasia and, on the contrary, new growth occurred as an entirely independent process.

The morphology of the tumors was constant in relation to the previous history of the breast. A distinctive papillary structure characterized all growths originating in cystic breasts while tumors arising in breasts with a normal antecedent history were distinguished histologically by an atypical proliferation of acini. The dependence of the papillar form of the disorder on preexisting cystic mastitis rather than on a special attribute of the family in which it occurs was apparently demonstrated by the fate of one of its members. In this animal, neoplasia was delayed for more than a year and a half beyond the group average but eventually occurred without preceding cystic change and was characterized morphologically by an acinar rather than by a papillar structure.

It appears, therefore, that while in certain animals cystic mastitis bears no relationship to the development of neoplasia and under certain conditions may occur as a non-progressive disease, it is, nevertheless, an essential precursor to one form of neoplasia in the rabbit. It is conceivable that similar considerations may apply to the association of these conditions in man.

The rôle of cystic disease in the pathogenesis of neoplasia is the subject of present investigations. A conception of the disease as an independent and etiologically unrelated process does not conform to known facts which, on the contrary, indicate a similar causal genesis. The possibility exists that animals of the family in question are distinguished by an inherent susceptibility to neoplasia which in the usual course of events is brought to expression by changes incident to a chance and causally unrelated attack

of cystic disease. If, on the other hand, cystic disease does not occur the susceptibility may still be expressed at a later date, as in the instance cited above. However, in view of the constancy with which cystic disease and neoplasia occurred as succeeding events in the breast, it seems more probable that they represent progressive stages of a pathological process. Other evidence indicative of such a relationship is found on examination of the endocrine organs of animals that died or were killed during different stages of the disorder.

The observation that the action of estrone extends beyond the organs of reproduction to embrace the entire endocrine system was first made by Cramer and Horning (1, 2) and has received abundant confirmation by other workers (3). The changes in the uterine mucosa, pituitary and suprarenal glands which follow long continued treatment with estrone are identical with those found in tumor-bearing rabbits of the family in question and, in view of the known carcinogenic action of such substances, particularly on mammary tissue, it is inferred that the spontaneous tumors represent a natural counterpart to the experimental production of neoplasia with such substances and arise on the same basis. In such cases, the presence of specific changes in the endometrium and endocrine glands in the earliest stages of cystic disease suggests the operation of the same factors in the genesis of that condition, while the progressive increase in the extent of the changes during subsequent stages of the disorder is additional evidence of a continuous disease process.

This conception may be extended to cover the second type of neoplasia as well as non-progressive cystic mastitis on the assumption of a differential reaction based on different genetic constitution or on variations in strength and duration of stimulus. Thus, cases of cystic mastitis which undergo regression may be considered as resulting from a temporary derangement of glandular mechanism of insufficient duration to produce secondary endocrine changes and withdrawal of the abnormal stimulation is followed by a return to normal breast structure. On the other hand, the adenomatous type of neoplasia without cystic disease but with comparable changes in the endometrium and in all endocrine organs, with the exception of the pituitary, may conceivably arise on the same basis in animals of fundamentally different constitution or at different age periods. The pronounced variation observed in the effects of estrone when administered to mice of different genetic constitution or of different ages bears on this point.

The progress of the disorder from non-invasive neoplasia to cancer was sometimes delayed for long periods of time and in a number of instances had not occurred at the time of death. In other individuals, cancer diag-

nosed on a basis of local invasion existed for many months as a relatively benign disease. It is of considerable interest that in many such cases foci of neoplasia were found in other organs and may have exerted an inhibitory influence on the mammary growth. Other animals were of an older age group or remained in poor physical condition and the progress of growth was similar to that which apparently occurs in tumors that form secondary autopsy findings in elderly human beings who have suffered from a long debilitating illness.

The coexistence of mammary and uterine tumors is not an uncommon finding in human pathology (4). In the present series of 25 mammary tumors, there were 6 coincident uterine growths of which one was a hemangioma and 5 were adenomata. This frequent association, together with the occurrence of mammary changes in nearly 50 per cent of cases of uterine adenomata, is additional evidence of the general involvement of the reproductive system in the disorder and is further suggestive of a hormonal origin. The occurrence of similar endocrine changes in animals with uterine and with mammary tumors indicates a common etiological factor, while the preponderance of breast growths in certain genetic lines and of uterine growths in others suggests the operation of accessory hereditary location factors.

SUMMARY

The clinical and pathological course of 25 mammary tumors in rabbits has been described. The antecedent breast history and morphology of the growths allowed a natural classification into two distinct types, one of which was distinguished by a preexisting cystic mastitis and a papillary structure, while the other originated in clinically normal mammary tissue and was characterized by an adenomatous structure. The two types of neoplasia occurred almost exclusively in two family groups and heredity played a fundamental rôle both in the occurrence of the tumors and in the determination of tumor type. Endocrine changes, comparable with those found in animals after long continued administration of estrogenic substances, occurred in tumor-bearing rabbits and it was inferred that the spontaneous growths represented a natural counterpart of the experimental induction of neoplasia with estrone.

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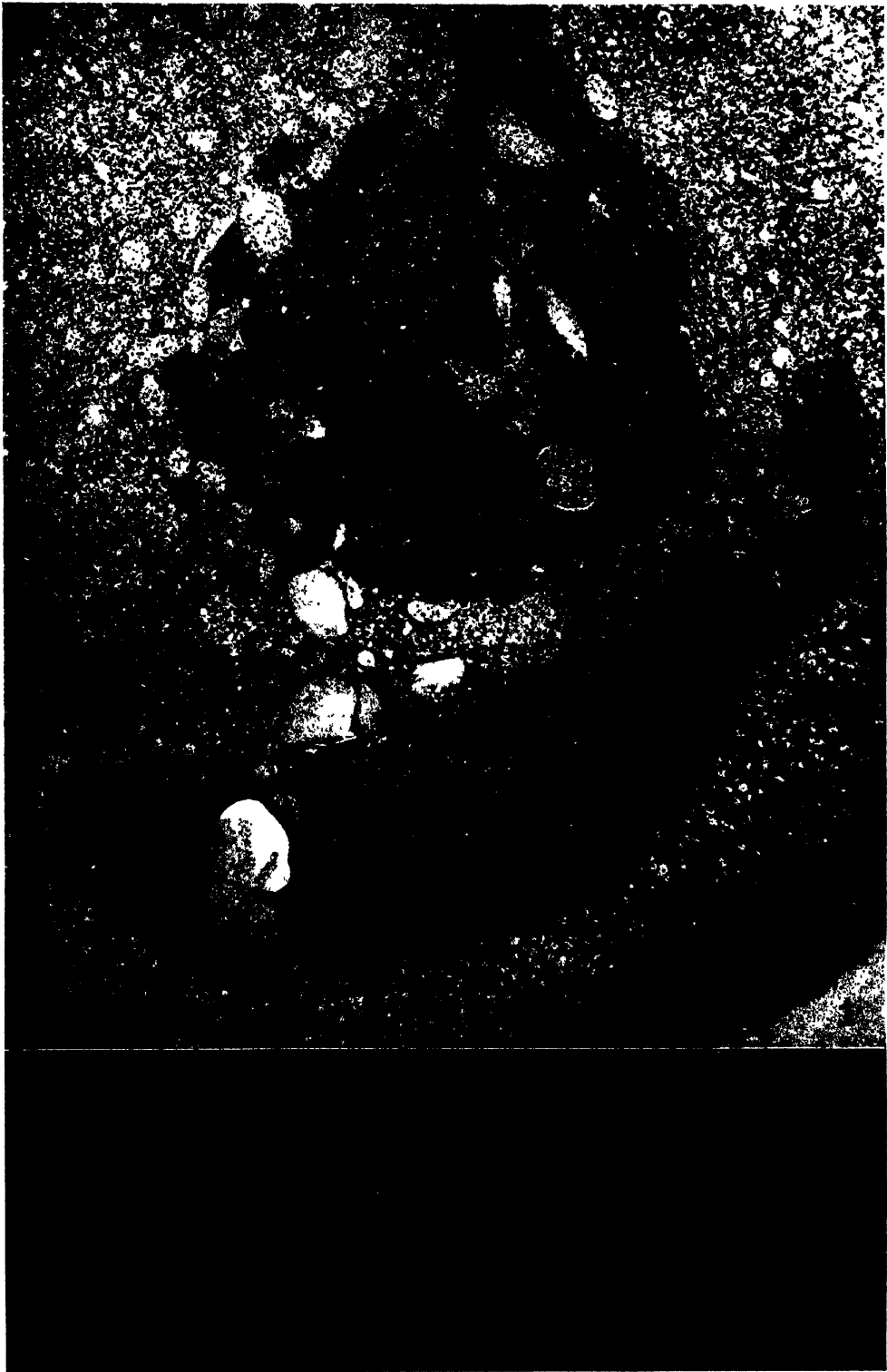
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EXPLANATION OF PLATES

PLATE 18

FIG. 1. Section of suprarenal. Greyish rounded areas containing large, pale cells are scattered throughout the cortex and medulla and tend to be concentrated in the boundary zone. In addition the cortex contains a number of cysts filled with homogeneous eosinophilic material. Hematoxylin and eosin. $\times 41$.

FIG. 2. Pituitary of a tumor-bearing animal compared with that of a normal animal of the same age, breed and size. The pituitary of the tumor-bearing animal weighed 308 mg., while that of the normal animal weighed 50 mg. $\times 1.55$.



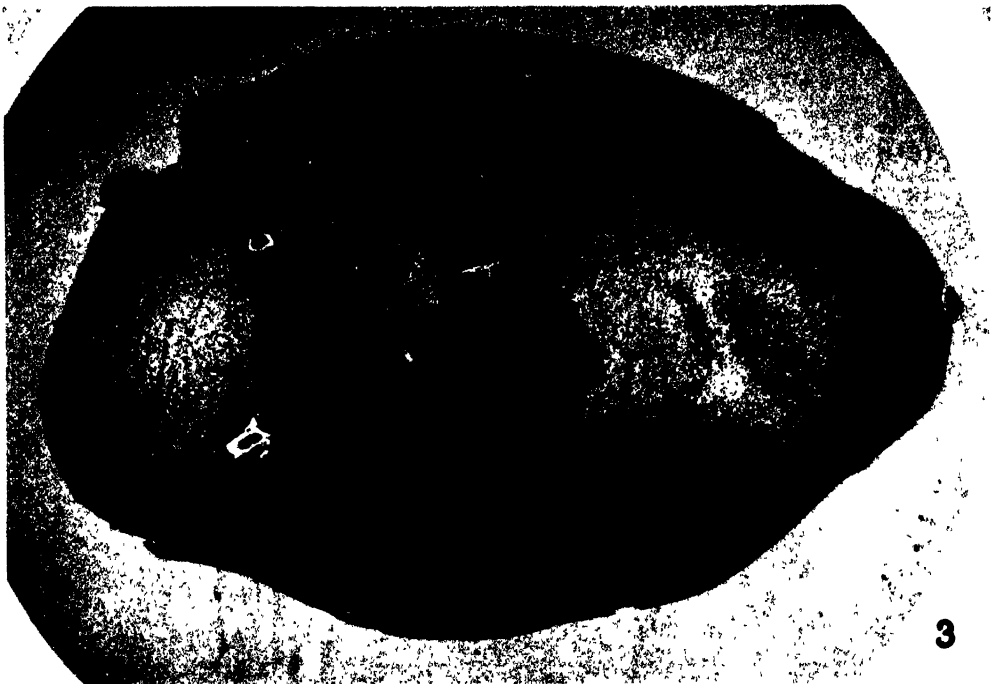
Photographed by J. A. Carlile

(Greene: Familial mammary tumors in rabbit. III)

PLATE 19

FIG. 3. Section of pituitary from a tumor-bearing animal showing a central, localized area of chromophobe cell hyperplasia. This is not usual and, as a rule, the chromophobe hyperplasia is generalized throughout the anterior lobe. Modification of Mallory's aniline blue. $\times 20$.

FIG. 4. Section of uterus from a tumor-bearing animal, showing areas of cyst formation and glandular hyperplasia. Hematoxylin and eosin. $\times 27.5$.



Photographed by J. A. Carlile

(Greene: Familial mammary tumors in rabbit. III)

FRENCHING OF TOBACCO

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Although frenching of tobacco was first described by John Clayton in 1688 (3) and has been recognized as a physiological disease since 1914 (2), the cause of the disease is still unknown. One hypothesis that has been advanced is that frenching may result from the toxic action of thallium. Several workers (4, 6, 1) have shown that the symptoms of thallium toxicity induced under controlled conditions simulate very closely those of natural frenching. Final proof that the disease is caused by thallium is lacking because it has been impossible to detect thallium in frenched plants or in field soil on which the disease occurs. Recent studies using the spectrograph are of interest in this connection.

Spectrographic analysis has been employed as a method for the detection of thallium in Turkish tobacco plants (*Nicotiana tabacum* L.). Approximately 25 mg. of dried plant material were placed on purified carbon electrodes and then arced with a direct current of 110 volts. The resultant spectra were photographed with a Hilger quartz spectrograph using 10-inch plates. The spectral line with a wave length of 2767.9 Ångstroms was found to be most useful for the detection of thallium. The results obtained with plants grown under greenhouse conditions are presented in table 1. These tests were first carried out in September, 1938, and later repeated in December, 1938, with similar results. Plants grown in nutrient sand cultures and supplied daily with a balanced, 3-salt nutrient solution at a concentration of 0.2 atmospheres gave a negative test for thallium. However, plants receiving the same nutrient solution together with sufficient thallium to produce only faint symptoms of toxicity (0.1 p.p.m. of thallium as $TlNO_3$) were able to absorb detectable amounts

of thallium from the solution. No thallium could be detected either in the tops of frenched plants grown in field soil or in healthy plants grown in composted soil.

These data suggest that frenching and thallium toxicity are two distinct physiological diseases. However, if the two diseased conditions are caused by different agents, it is surprising that they are so similar not only with respect to the symptoms produced on Turkish tobacco, but also with regard to the methods by which they are controlled (5, 6). Furthermore, a water extract of a non-toxic soil, collected near a natural frenching area, does not produce frenching

TABLE 1

Spectrographic Analysis of Thallium in Tobacco Plants Receiving Various Amounts of Thallium

Plants grown in	Thallium added (p.p.m.)	Disease symptoms	Spectrographic test
Sand.....	0	None	—
"025	None	—
"05	Only lower leaves slightly yellow	—
"1	Faint mottling throughout plant	+
"2	Slight strap-leaf formation	+
Field soil.....	0	Chlorosis typical of frenching	—
Composted soil..	0	None	—

until supplemented by the additive effect of a non-toxic amount of thallium (6). Root symptoms of both diseases are also similar (5, 6).

It is not improbable that the toxic action of thallium may be exerted on the root, thereby altering its metabolism, and giving rise to some disturbance that affects the top. Since it is not known in what form thallium may occur in the soil, it is conceivable that it may be present as some complex compound that is not so readily translocated into the top as the thallium salts used in experimental studies. If this were true, the spectrographic detection of thallium only in those plants treated with $TiNO_3$ would not necessarily eliminate the possibility that thallium may be the cause of frenching.

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